ABC transporters in insect detoxification pathways

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by Dipl-Biol. Anne Bretschneider
born on 29.04.1986 in Berlin
(born: Anne Karpinski)

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Gutachter: 1. Prof. Dr. David G. Heckel, Max-Planck-Institut für Chemische Ökologie, Jena

2. Prof. Dr. Ping Wang, Cornell University, Ithaca, USA

3. Prof. Dr. Stefan H. Heinemann, Friedrich-Schiller-Universität, Jena

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Abbreviations

The abbreviations used in the manuscripts are mentioned below the respective abstract.

ABC	ATP-binding-cassette (transporter)
ATP	adenosine triphosphate
bp	basepair
Bt	*Bacillus thuringiensis*
Cry	crystal protein / crystal toxin derived from *Bacillus thuringiensis*
GST	glutathione S-transferases
HevABCC2	ABC transporter C2 from *Heliothis virescens*
HevCaLP	cadherin-like protein from *Heliothis virescens*
kDa	kilo Dalton
NBD	nucleotide binding domains
OGS	official gene set
P450	cytochrome P450
qRT-PCR	quantitative real-time polymerase chain reaction
RNAseq	RNA-Sequencing
Sf	*Spodoptera frugiperda*
TM	transmembrane domain
UGT	UDP-glycosyl transferases

1. Introduction

The class of insects comprises the largest number of species on earth and almost half of these feed on plants.[1] However, plants are not unprotected and their defense mechanisms are of both physical and chemical nature.[2-4] This led to a co-evolutionary arms race of defense mechanisms in plants and counterdefenses or adaptive mechanisms in insects.[2, 5, 6] While this process may be disturbed by external factors, for example the use of insecticides in agriculture, insects possess a suitable instrument to overcome both obstacles, plant defensive compounds as well as certain insecticides: ATP-binding cassette transporters.

1.1 An important tool for detoxification: The multigene family of ATP-binding cassette transporters

ATP-binding cassette (ABC) transporters are transmembrane proteins, which actively transport molecules across a biological membrane by hydrolyzing adenosine triphosphate (ATP).[7] These genes are present in all organisms from bacteria to human and perform either import or export, but never symport.[8] The first ABC transporter ever to be discovered, was the histidine transporter in *Salmonella typhimurium*.[9]

A functional eukaryote ABC transporter, which performs export, consists of four core domains: two membrane spanning domains (transmembrane domain, TM), each built up from six membrane spanning α-helices, alternating with two nucleotide binding domains (NBD) located on the cytosolic side (see **Fig. 1.1 A**).[7, 10] All four domains may be fused into a single polypeptide (full-transporter) or a functional ABC transporter may also be achieved by dimerization of two half-transporters, each consisting of one NBD fused to one TM.[11, 12] Yet, there are also exceptions, like an additional TM or the absence of both TMs.[8, 13]

Even though different ABC transporters show little sequence homology regarding their TMs, their NBDs are highly conserved, but not invariant. ABCs comprise seven distinct sequence motifs: the A-, Q-, D- and H-loop, as well as the Walker A and B motifs and the so called ABC signature (LSGGQ).[14] These motifs are directly involved in the binding of ATP during the transport mechanism, which comprises four distinguishable steps (see **Figure 1.1 B**).[7, 14-16] A ligand binds into a cavity between the TMs, which causes a conformational change, bringing the NBDs in close proximity. This facilitates

the binding of two adenosine triphosphate (ATP) molecules. Subsequently, the ligand is released into the extracellular space. In a final step the ATP molecules are hydrolyzed and released as adenosine diphosphate (ADP) and inorganic phosphate into the cytosol. ABC transporters perform different functions within the cell, such as the transport of lipids and inorganic ions, detoxification of xenobiotics as well as protein synthesis.[8] However, the subfamily classification (from A to H) is based on the structure. The subfamily H has just recently been discovered in invertebrates, but its function is still unknown.[17]

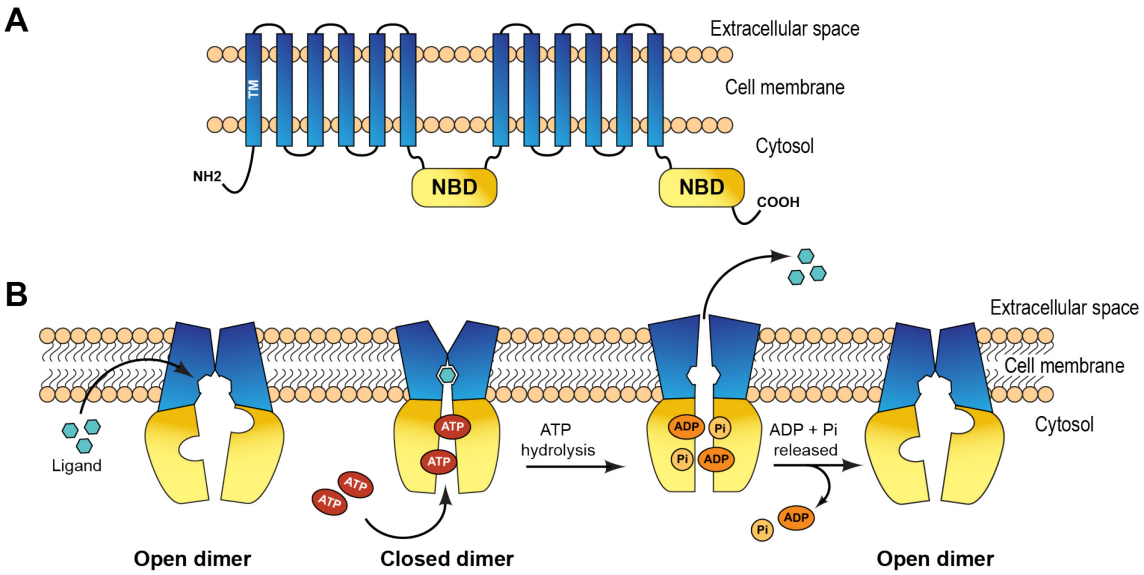

Figure 1.1| Structure and transport mechanism of ATP-binding cassette (ABC) transporters. (A) The core structure of a full ABC transporter, which is required for export, consists of 12 membrane spanning α-helices organized in two transmembrane domains (TM) and two nucleotide binding domains (NBD). (B) An ATP-switch mechanism drives substrate transport (adapted from [7]).

Members of the ABCA subfamily are the largest identified ABC genes, encoding for over 2100 amino acids.[8] These genes are involved in cell and body lipid homeostasis.[18] The subfamilies ABCB and ABCC are associated with the phenomenon of multidrug resistance, "where resistance developed to one type of xenobiotic gives resistance to a different class of xenobiotic".[19] In *Trichoplusia ni* members of both families are highest expressed in Malpighian tubules, one of the main excretory organs.[20, 21] P-glycoprotein (Pgp, ABCB1) was the first cloned human ABC transporter and it was shown to transport several hydrophobic substrates, such as colchicine and vinblastine.[22, 23] The ABCC subfamily is functionally quite diverse, as it contains the chloride channel

involved in cystic fibrosis, the membrane-bound sulfonylurea receptors, as well as the multidrug resistance-associated proteins.[24] Members of the ABCD subfamily have been associated with the regulation of very long chain fatty acid transport and adrenoleukodystrophy.[25, 26] Across the class of insects the number of genes in this subfamily is conserved (**Table 1.1**). The subfamily ABCE is small and comprises just a single gene: ABCE1 (in mammals also known as RNase L inhibitor).[27] RNA interference studies in *Caenorhabditis elegans* confirmed the involvement of ABCE1 in gene transcription and translation, as well as the shuttling of proteins between the nucleus and the cytoplasm.[27] Studies on ABCF1 in *Saccharomyces cerevisiae* and *Homo sapiens* revealed its association with the ribosome.[28, 29] Members of both families have no TM and are therefore not involved in membrane transport mechanisms.[8] The subfamily ABCG contains both full- and half-transporters, which are involved in cholesterol transport in mammals.[30, 31] However, ABCG2 was also shown to confer drug resistance by transporting anthracyclines across the placenta.[32, 33] These examples from different subfamilies illustrate that ABCs are involved in many cellular processes.

Table 1.1| Comparison of gene numbers in the different ABC transporter subfamilies of six arthropod species (*Bombyx mori*, *Drosophila melanogaster*, *Tribolium castaneum*, *Chrysomela populi*, *Tetranychus urticae*, *Daphnia pulex*) and *Homo sapiens*.[8, 18, 24, 34-40]

Subfamily	*B. mori* [a]	*D. melanogaster*	*T. castaneum*	*C. populi*	*T. urticae*	*D. pulex*	*H. sapiens* [b]
A	9	10	10	5	9	4	12
B	9	10	6	8	24	7	11
C	15	12	35	29	39	7	12
D	2	2	2	2	2	3	4
E	1	1	1	1	1	1	1
F	3	3	3	3	3	4	3
G	13	15	13	14	23	24	5
H	3	3	3	3	22	15	-
Total	**55**	**56**	**73**	**65**	**103**	**65**	**48**

[a] Numbers represent the highest identified number of genes for the respective subfamily.[39, 40]
[b] *Homo sapiens* do not possess ABC transporters of the subfamily H.

The number of identified ABC genes differs widely among species (**Tab. 1.1**).[37] While *H. sapiens* possesses 48 ABC transporters, 82 members of this gene family were identified in the Lepidopteran *Plutella xylostella*, and 105 were found in the genome of the spider mite *Tetranychus urticae*.[18, 35, 41] Most of the research on ABC transporters has been done in bacteria and vertebrates, focusing on multidrug resistance and diseases. However, although information about ABC transporter functions in invertebrates is generally quite scarce, there are a few notable exceptions. In the poplar

leaf beetle *Chrysomela populi* it was shown for the first time that ABC transporters are involved in sequestration, a strategy to detoxify xenobiotics.[42] Strauss et al. showed that a member of the ABCC subfamily transports salicin, a secondary metabolite present in the beetle's host plants.[42] The *white* locus of *Drosophila melanogaster* is the most intensively studied ABCG gene and has even become an important marker in fly genetics. Together with *brown* and *scarlet*, it is involved in the transport of the eye pigment precursors [43], which also demonstrates that the vertebrate function (cholesterol transport) may not always be applicable to invertebrates [30, 31], if solely based on subfamily affiliation. Furthermore, ABC transporters confer insecticide resistance.[19, 37] In *Helicoverpa armigera* the ABCB1 protein exports Fenvalerate and Cypermethrin from the cell.[44-46] In addition, an age-dependent increase in Thiodicarb resistance in *Heliothis virescens* was linked to ABCB1 as well.[47, 48]

1.2 Insect-plant interactions

Plants have developed a vast amount of chemical defenses against insect herbivores.[4] These defenses comprise repellents, antifeedants and toxins, as well as volatiles for the attraction of predators.[49, 50] Most of these plant defenses are based on the production of secondary metabolites, organic compounds with no direct influence on the growth, development and reproduction of the plant.[51] Known plant insecticidal toxins are alkaloids (e.g. pyridine and pyrrolizidine derivatives), terpenoids (e.g. cardenolides) and phenolics (e.g. tannins).[52-55] Yet, insects have adapted to these toxins and developed countermeasures to overcome these barriers by investing into detoxification mechanisms.[3]

Across all herbivorous insects, most have specialized on a few host plants, respectively one or two related plant families, while less than 10% are generalists, feeding on a wide variety of plant families.[56, 57] However, even though they are called generalists, the latter show preferences for certain host plants as well.[58] Specialists may encounter a range of rather uniform compounds, including a more narrow range of secondary metabolites. Therefore, in cases where specialist herbivores evolved efficient and constitutive defense mechanisms, they can maximize development.[50] Generalists on the other hand are thought to possess many different biochemical defense strategies at the cost of lower feeding success.[59] Yet, the different toxins also elicit the requirement for regulatory mechanisms in the insect to induce an adequate defense strategy.[50]

1.2.1 Detoxification of plant secondary metabolites

Plant insecticidal toxins are rapidly detoxified by sequestration, metabolization into non-toxic compounds or excretion.[50] Many aposematic specialist butterflies and diurnal moths, but also beetles, perform sequestration to avoid deleterious effects from their host plants and at the same time to be protected against predators.[60, 61] As prominent example, the six-spotted burnet moth *Zygaena filipendulae* sequesters cyanogenic glucosides, which are acquired from the host plant *Lotus corniculatus* or by *de novo* biosynthesis.[62, 63]

Another detoxification strategy is the metabolization into non-toxic compounds. Cytochrome P450s (P450) are membrane-bound enzymes with the ability to metabolize xenobiotics.[64] Recent studies have underlined their role in host plant adaptation. The silencing of a P450 (CYP6AE14) in *H. armigera* was accompanied by retarded larval growth when fed on gossypol, a secondary metabolite present in cotton.[65, 66] Similarly, the black swallowtail *Papilio polyxenes*, which feeds exclusively on furanocoumarin containing plants, utilizes P450s for detoxifying host plant derived secondary metabolites.[67] This species harbors two specialized P450s (CYP6B1 and CYP6B3), which enable *P. polyxenes* to efficiently detoxify these furanocoumarins.[67, 68] These examples reveal a direct role of cytochrome P450s in detoxification of secondary metabolites and their role in host plant adaptation.

Another gene family, the glutathione S-transferases (GST), is often associated with the detoxification of xenobiotics as well.[69, 70] These enzymes catalyze the conjugation of lipophilic compounds to reduced glutathione (GSH) and their activity is induced through secondary metabolites.[71] In *Manduca sexta* it was shown that there was GST gene upregulation in response to plant feeding, yet the response was not specific.[70] Furthermore, three GSTs were upregulated in *H. armigera* in response to feeding on gossypol.[72] GSTs are therefore another important gene family which enables insects to cope with the chemical defenses of their host plants.

Xenobiotics may also be detoxified by conjugation to a sugar molecule, an enzymatic activity found in the UDP-glycosyl transferase (UGT) gene family.[73] The conjugation of xenobiotics with a sugar molecule enhances their solubility in water and therefore the excretion. The two closely related lepidopteran species *Helicoverpa assulta* (specialist) and *H. armigera* (generalist) can both tolerate high concentrations of capsaicin and both

utilize UGTs for detoxification of this host plant-derived compound.[50, 74] The glucoside is metabolized in the Malpighian tubules and the fat body and afterwards excreted into the feces.[75]

A number of studies have identified highly adapted insects which feed with impunity on their host plants, but are not relying on enzyme activities from known detoxification-related gene families. One way of acquiring new detoxifying genes is by evolutionary recruitment.[6, 73] For example, the European cinnabar moth *Tyria jacobaeae* detoxifies the pyrrolizidine alkaloids from their host plant *Senecio jacobaea* by N-oxidation.[76] The respective gene, a flavin-dependent monooxygenase, was acquired by gene duplication and neofunctionalization.[77] The same was observed in the butterfly family Pieridae. These insects have specialized on brassicaceous plants, and are the only known species to possess nitrile-specifier proteins (NSPs), key elements for glucosinolate detoxification and adaptation to their host plants.[64, 78] Furthermore, a glucosinolate sulfatase (GSS) has been identified in *Plutella xylostella*, also a specialist on glucosinolate-containing plants, which prevents the formation of toxins.[79] The NSP and GSS arose through gene duplication in combination with neofunctionalization. The results were novel functions that the parental genes do not harbor.[64]

Apart from sequestration and metabolization, xenobiotics may also be rapidly excreted.[1, 80] This adaptation mechanism was first shown in *M. sexta*.[81, 82] Larvae of this species rapidly excrete nicotine and other ingested alkaloids (up to 93%) together with the feces to avoid intoxication.[82] In contrast, the house fly *Musca domestica* neither excreted nicotine nor other alkaloids in similar amounts.[81] The small remaining amount of nicotine in *Manduca* larvae is excreted via the Malpighian tubules. However, it is still unknown how nicotine is transported from the hemolymph into these excretory organs. Several studies have proposed the involvement of ABC transporters or ABC transporter-like proteins, suggesting a similar mechanism as in plants.[83-85] Once again, little is known on ABC transporter involvement in insect-plant-interactions.

1.2.2 Identification of detoxification related genes: Next generation sequencing

More recent molecular methods, such as the utilization of next generation sequencing, enable researchers to perform a broad screening for adaptive gene regulation. One of these methods is the transcriptome analysis using RNA-Sequencing (RNAseq). This

method offers several advantages compared to microarrays and quantitative real-time PCR (qRT-PCR).[86, 87] It is not limited to existing genomes, which is of importance when working with a non-model organism.[88] Furthermore, it shows rather low background and is, when performed correctly, highly accurate; however most importantly it allows a qualitative and quantitative analysis of transcriptional differences between samples.[86, 87]

Studies in insects, which apply RNAseq, have focused on different aspects of insect biology [38, 41, 89-91], yet the overall number of available studies on host plant adaptations is still scarce. One such study, a transcriptome analysis utilizing RNAseq in the generalist *T. urticae*, revealed differential ABC transporter expression when fed on different host plants.[35] These results allow a fast and comprehensive analysis of insect adaptation mechanisms and the identification of candidate genes, including in particular the ABC transporter gene family.

1.2.3 Study organisms: Two generalists and a specialist

The *Heliothinae* subfamily within the Noctuidae (Lepidoptera) comprises several agricultural pest species. Two of these pests are the cotton bollworm *Helicoverpa armigera* and the tobacco budworm *Heliothis virescens.*[92, 93] Both species are polyphagous.

Many studies have focused on the life history traits of *H. virescens*, especially host plant choice, sexual communication and the immune system.[58, 94, 95] This species is one of the major pests on cotton (*Gossypium hirsutum*) and tobacco (*Nicotiana tobaccum*) in the United States.[96, 97] Beyond that, *H. virescens* feeds on plants belonging to more than 37 species from 14 different families across North and South America.[92, 98] This very broad host plant range requires this species to adapt to very different plant defense mechanisms.[99-101] Adding to this, *Heliothis* has also evolved resistance to many synthetic insecticides, such as dichlorodiphenyltrichloroethane (DDT) and pyrethroids.[47, 48, 102-105] All of these factors make *H. virescens* a suitable species to study insecticide resistance.

The closely related species *H. armigera* was originally native to Eurasia, Africa and Oceania. However, just recently this species has been accidentally introduced to South America.[97, 106, 107] The polyphagous larvae (up to 40 host-plant species are reported) feed for example on cotton, tobacco, corn (*Zea mays*) and sunflower (*Helianthus*

annuus), four plants with different chemical defenses.[92, 108] Studies on the transcriptome of *Helicoverpa* showed a host-plant specific response, for example when larvae were fed on tobacco and cotton.[109] Known responses to secondary metabolites comprise the upregulation of P450s, GSTs, serine proteases and the regulation of amylase activity.[72, 110, 111] In addition, this species has also developed insecticide resistance to pyrethroids in the field.[112, 113] The fact that *H. armigera* encounters such diverse plants, allows comprehensive studies on its plant adaptation mechanisms.

The tobacco hornworm *Manduca sexta*, a member of the Sphingidae (Lepidoptera) present throughout North and South America, shows a specialized lifestyle compared to *H. virescens* and *H. armigera*. *Manduca* larvae feed on plants belonging to the nightshade family (Solanaceae). Nevertheless, it has been reported that larvae are also able to develop on non-host plants such as *Brassica* spp.[70, 114-116] Common host plants are coyote tobacco (*Nicotiana attenuata*), tomato (*Solanum lycopersicum*) and *Datura wrightii*.[114, 117, 118] *Manduca* has become a model species for lepidopteran insects, not least because of many studies on its behavior, immunity, olfaction and biochemistry.[119-121]

1.3 Insecticide resistance: The special case of Bt toxins

There is a growing demand for insecticides in agriculture. However, the usage of chemical substances throughout the last decades led to the development of resistance and, as a consequence, also to the discovery of alternatives.[122, 123] The most widely used insecticides nowadays are obtained from the gram-positive bacterium *Bacillus thuringiensis*, known as Bt. These insecticides have important properties which make them highly attractive for insect control: i) they are host specific, ii) environmentally benign and iii) can be used as foliar sprays or by expressing the respective gene in transgenic crops.[124, 125]

Bacillus thuringiensis produces different proteins with insecticidal activity, e.g. the cytolytic proteins (Cyt) and the vegetative insecticidal proteins (Vip).[126] These toxins were shown to be active against Diptera and Lepidoptera.[127, 128] However, the biggest group of insecticidal proteins are the crystal proteins (Cry) [129], which are produced during sporulation and stored in crystalline inclusions. Over 700 Cry proteins have been identified in *Bacillus* so far.[130] Since the amount of identified insecticidal proteins continuously increases, a four-rank nomenclature system was established based on

sequence similarity.[126] For example Cry1Aa, Cry1Ab and Cry1Ac share between 78% and 95% pairwise identities. A special feature about Cry toxins, which also explains their massive use in agriculture, is their host specificity. To date, Cry toxins were discovered which are, among others, active against Lepidoptera (moth and butterflies), Diptera (flies and mosquitoes), as well as Hymenoptera (wasps) and Coleoptera (leaf beetles and weevils).[131-138] This specificity arises through differences in the proteolytic activity between the species and the interaction with different receptors.[139, 140] At the same time, this specificity is a big advantage for using Bt toxins, because there are low off-target effects.[141] It has been shown for example that Cry1B is toxic to lepidopterans, but not to coleopterans, except if the toxin has been previously solubilized.[142]

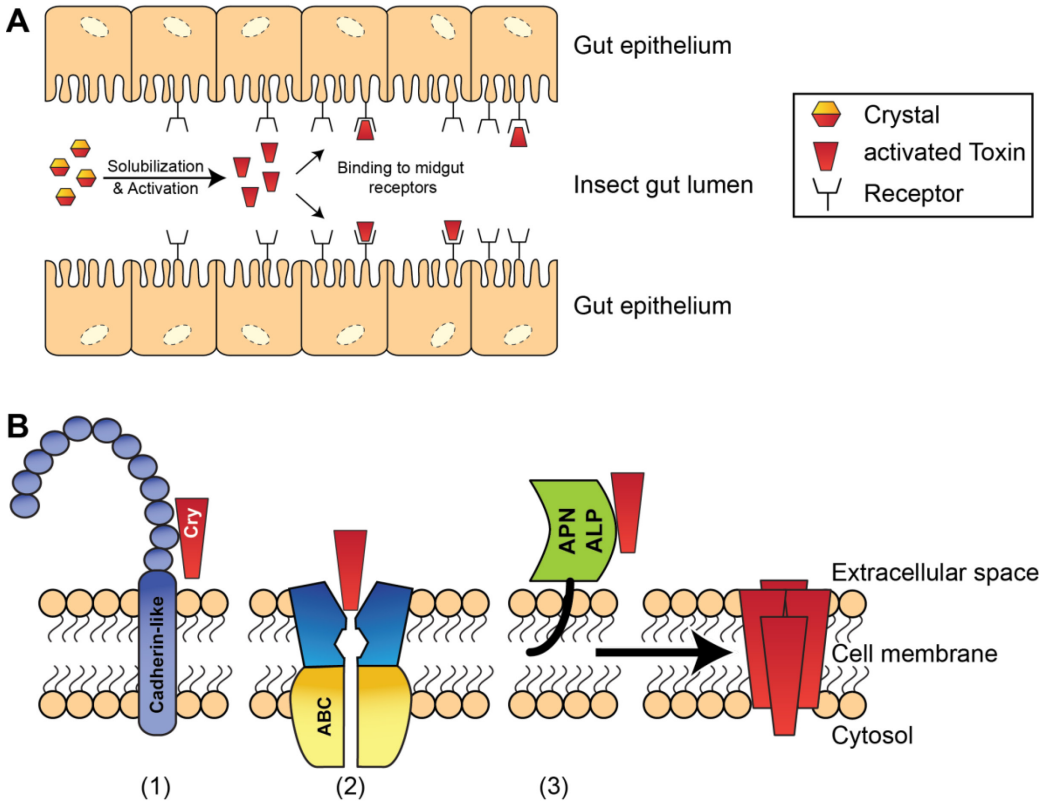

Figure 1.2| Mode of action of Cry1A toxins. (A) *Bacillus thuringiensis* (Bt) crystals are ingested and solubilized in the midgut. The activated 65 kDa toxin (shown in red) binds to receptors of the midgut cell membrane. (B) A cadherin-like protein (named HevCaLP in *H. virescens*) (1), an ABC transporter (2), ALP and APN (3) have been proposed to function as Cry1A toxin receptors. The interaction with either one or more leads to toxin oligomerization, membrane insertion and eventually to pore formation. This will lead to cell lysis and cell death, which in turn will damage the epithelium and lead to insect death.

Different models have been proposed for the mode of action leading to insect death.[143] Nevertheless, all these models have in common that the toxin interacts with membrane bound receptors (**Figure 1.2**).[144] More and more receptors have been proposed; however

this aspect has not been fully resolved yet.[145-149] The first Cry1A receptor, which has been identified by the use of binding studies, showed sequence similarity to the Cadherin-superfamily in *M. sexta*, and was therefore named cadherin-like (CaLP).[150, 151] Further experiments support these finding, e.g. it was shown that the disruption of the cadherin gene (named HevCaLP) was genetically linked to Cry1Ac resistance in laboratory *H. virescens* populations.[145] The cellular function of the protein has not been discovered yet. In addition, alkaline phosphatase (ALP) and aminopeptidase N (APN) have been proposed to function as receptors for Cry1A toxins.[147, 148, 152] ALP performs a hydrolytic function within the cell and APN plays a multifunctional role, e.g. cell adhesion and peptide metabolism.[153, 154] During the last years, ABC transporters played an increasing role as Bt toxin receptors, with ABCC2 in *H. virescens* leading the way.[155, 156] Four laboratory strains of *Heliothis* showed different Cry1Ac resistance levels, which were coupled to the expression of ABCC2. A closely related ABC transporter, ABCC3, was suggested to be a receptor for Cry1Ca in *Spodoptera exigua* larvae.[149]

The "classical model" is the oldest to explain the Cry toxin mode of action, yet the least resolved.[143, 157] Subsequently after the ingestion by the insect, the crystal is solubilized in the midgut, releasing a protoxin (δ-endotoxin, 130 kDa) which is activated by the alkaline pH and midgut proteases.[140] The activated 65 kDa toxin, which contains three domains, then interacts with receptors on the surface of the gut epithelium.[140] This leads to insertion into the membrane and pore formation, causing a disruption of the membrane integrity and eventually leading to insect death after a few days.[143, 157, 158] The "sequential binding" model proposes a "ping-pong" mechanism where the activated toxin interacts with a glycosylphosphatidylinositol (GPI)-anchored APN, before binding to the cadherin-like protein.[159] The domains II and III of the Cry toxins recognize and bind the receptor.[140, 159, 160] This leads to the oligomerization of the toxin monomers and the formation of a "pre-pore" structure, which binds again to the APN and finally will be inserted into the membrane.[148] For this step the presence of domain I of the toxin is crucial.[140] The "signaling pathway" model uses another approach to explain the Cry toxin mode of action. Upon binding to the cadherin protein, the Mg^{2+}-dependent protein kinase A signaling pathway is initiated that leads to necrotic cell death.[161] It can be concluded that some steps of these models are quite well supported, while others still need further evidence.[143]

The first cases of resistance to Cry toxins have been observed in field populations of Lepidoptera and Coleoptera since the end of the 20th century, for example *Plutella xylostella*, *Trichoplusia ni*, *Busseola fusca*, *Diabrotica virgifera virgifera*, *Spodoptera frugiperda*, and *Pectinophora gossypiella*.[124, 131, 137, 162-168] Here, resistance is the "genetically based decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin in the field".[168] *P. xylostella*, an agricultural pest of cruciferous plants, was the first species to show field-evolved resistance towards Bt treatment.[131] Laboratory bioassays revealed a higher lethal concentration (LC_{50}) of a field population in comparison to a susceptible laboratory population. Remarkable is also the discovery of a cross-resistance between Cry3Bb1 and mCry3A in field populations of *D. v. virgifera*, which is a key pest of maize.[137] Many studies have identified a genetic basis as the main cause for resistance.[124, 149, 169] For example, in *H. virescens* the expression of HevCaLP and the ABC transporter HevABCC2 were shown to significantly influence the survival of larvae on Cry1Ac as well as the Cry toxin binding to brush border membrane vesicles.[145, 156] Furthermore, the field-evolved resistance in *P. xylostella* was shown to have a genetic basis which was independent of the cadherin-like protein.[124] The Cry1Ac resistance in a greenhouse population of the cabbage looper *T. ni* was associated with the loss of binding to the midgut, implicating a missing interaction with a receptor.[132]

These cases of resistance have evolved in a short period of time and the reason was a selection pressure through the constant usage of Bt foliar sprays, as well as the introduction of transgenic plants.[168] Therefore the "high-dose/refuge resistance management strategy" was proposed to delay the evolution of insecticide resistance.[170] This requires the presence of untreated or non-transgenic plants next to plants treated with a high dose of Bt toxins. The untreated plants will serve as a refuge for susceptible insects, which will mate with resistant ones. The resulting heterozygous offspring will eventually be susceptible to the toxins.[170] Studies on *P. gossypiella*, *H. virescens*, *H. armigera* and *Helicoverpa punctigera* have demonstrated that Bt resistance evolved more slowly when this strategy is applied.[168]

1.4 Aim of this thesis

The multigene family of ATP-binding cassette (ABC) transporters has been shown to be involved in xenobiotic detoxification, as well as conferring multidrug resistance. These transmembrane proteins have been thoroughly investigated in vertebrates and bacteria, yet the information in insects is still scarce. Herbivorous insects face a complex set of defense mechanisms, especially secondary metabolites which are repellent or toxic. In addition, herbivorous insects encounter insecticides which are used to protect the plant. Different projects throughout this thesis were realized to investigate ABC transporters in lepidopteran insects and their role in detoxification pathways, as well as their importance for the mode of action of known insecticides.

In order to investigate the latter, two putative receptors for *Bacillus thuringiensis* (Bt) toxins from *Heliothis virescens* were heterologously expressed in an insect cell line (**Chapter 2.1**). The toxin-receptor interaction was analyzed by binding studies and competition assays. Time lapse recordings were performed to confirm the results of a toxicity assay. To rule out the involvement of endogenous receptors expressed in the cell line, transcript levels were analyzed with qRT-PCR. In addition, *in vivo* toxicity assays were performed to endorse the *in vitro* results. A transcriptome analysis was performed to explore how xenobiotics elicit changes in ABC transporter gene expression in the generalist *Helicoverpa armigera* (**Chapter 2.2**). A replicated RNAseq approach was combined with the official *Helicoverpa* gene set. Gene expression changes were characterized in different developmental stages, as well as larval tissues. The influence of secondary metabolites on larval development was recorded to test for any detrimental effects, alongside of the influence on ABC transporter gene expression. **Chapter 2.3** discusses the influence of host plant feeding on the transcriptome of *Manduca sexta*, a specialist on solanaceous plants. A special focus was put on immune system-, detoxification- (e.g. ABC transporters) and olfaction-related genes since they resemble three important aspects of host plant adaptation. A replicated RNAseq study was performed together with a developmental assay to compare larval response between host- and non-host plants. The ABC transporters for *H. armigera* and *M. sexta* were annotated beforehand (**Chapter 3**).

The results of this thesis are discussed in the context of insect adaptation mechanisms, focusing on the role of ABC transporters in plant secondary metabolite detoxification as well as insecticide resistance.

2. Manuscripts

Manuscript I (Chapter 2.1)

Three toxins, two receptors, one mechanism: Mode of action of Cry1A toxins from *Bacillus thuringiensis* in *Heliothis virescens*

Anne Bretschneider, David G. Heckel, Yannick Pauchet

Manuscript submitted on December 10th to

PLOS Pathogens

The Manuscript I investigates the role of the ABC transporter HevABCC2 in the *Bacillus thuringiensis* (Bt, Cry1A) toxin mode of action in *Heliothis virescens*, as well as its interaction with the protein HevCaLP (a cadherin-like protein). These two putative receptors were heterologously expressed in Sf9 insect cells, deriving from *Spodoptera frugiperda*. By the use of *in vitro* toxicity assays, binding studies as well as time lapse recordings, it was shown that HevABCC2 is the main target for the Cry1A toxins (Cry1Aa, Cry1Ab, Cry1Ac) and that HevCaLP plays a supporting role by enhancing the mode of action. Based on these results a new model for the mode of action of Cry1A toxins is proposed.

Anne Bretschneider and Yannick Pauchet planned the experimental outline. Anne Bretschneider established and performed the experiments and prepared all figures. With the support by Yannick Pauchet, Anne Bretschneider analyzed the data. Anne Bretschneider wrote the manuscript, which was revised by Yannick Pauchet. David G. Heckel participated in the design and coordination of this study and helped with writing the manuscript.

Manuscript II (Chapter 2.2)

Know your ABCs: Characterization and gene expression dynamics of ABC transporters in the polyphagous herbivore *Helicoverpa armigera*

Anne Bretschneider, David G. Heckel, Heiko Vogel

Manuscript submitted on November 30th to

Insect Biochemistry and Molecular Biology

Manuscript II is a study on the multigene family encoding for ABC transporters in the polyphagous moth *Helicoverpa armigera* (Noctuidae). RNA-Sequencing (RNAseq) revealed specific expression profiles in different life stages and different tissues. The feeding of host plant and non-host plant derived secondary metabolites altered larval development as well as ABC transporter expression. The results illustrate that this polyphagous species exhibits general detoxification mechanisms. The results were discussed in the context of detoxification and insect-plant-adaptation.

Anne Bretschneider and Heiko Vogel planned the experimental outline. Anne Bretschneider performed and established the experiments, analyzed the data with the help of Heiko Vogel and prepared all figures. Anne Bretschneider wrote the manuscript. David G. Heckel participated in the coordination of this study. Heiko Vogel and David G. Heckel revised the manuscript.

Manuscript III (Chapter 2.3)

The plastic response of *Manduca sexta* to host and non-host plants

Christopher Koenig, Anne Bretschneider, David G. Heckel, Ewald Grosse-Wilde, Bill S. Hansson, Heiko Vogel

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In Manuscript III, the model organism *Manduca sexta* was tested for its adaptive responses to secondary metabolites in its host plants. The larval performance and the transcriptional response (RNAseq) to host plants and non-host plants were analyzed. Surprisingly, *M. sexta* larvae performed equally well on solanaceous host plants as well as the non-host plant *Brassica napus*. However, larvae exhibited a transcriptional response specific for each plant, such as specific changes in the expression of genes related to detoxification, for example ABC transporters. This provides insights into the plastic response of an herbivorous insect with a restricted repertoire of host plants.

Anne Bretschneider and Christopher Koenig share the first authorship.

Anne Bretschneider, Christopher Koenig and Heiko Vogel planned the experimental outline. Anne Bretschneider and Christopher Koenig analyzed the data, prepared the figures and wrote the manuscript. Anne Bretschneider performed the statistical analysis. Heiko Vogel, David G. Heckel, Ewald Grosse-Wilde and Bill S. Hansson participated in the coordination of this study and revised the manuscript.

2.1 Manuscript I

Three toxins, two receptors, one mechanism: Mode of action of Cry1A toxins from *Bacillus thuringiensis* in *Heliothis virescens*

Anne Bretschneider [1], David G. Heckel [1], Yannick Pauchet [1]

[1] Department of Entomology, Max-Planck Institute for Chemical Ecology, Jena 07745, Germany

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Abstract

Insecticidal crystal (Cry) proteins from *Bacillus thuringiensis* (Bt) are highly active against Lepidoptera. However, field-evolved resistance to Bt toxins is on the rise. The 12-cadherin domain protein HevCaLP and the ABC transporter HevABCC2 are both genetically linked to Cry toxin resistance in *Heliothis virescens*. We investigated their interaction using stably expressing non-lytic clonal Sf9 cell lines expressing either protein or both together. Untransfected Sf9 cells are innately sensitive to Cry1C toxin, but not to Cry1A toxins; and quantitative PCR revealed negligible expression of genes involved in Cry1A toxicity such as cadherin, ABCC2, alkaline phosphatase (ALP) and aminopeptidase N (APN). Cry1Aa, Cry1Ab or Cry1Ac caused swelling of Sf9 cells expressing HevABCC2, and caused faster swelling, lysis and up to 86% mortality in cells expressing both proteins. No such effect was observed in control Sf9 cells or in cells expressing only HevCaLP. The results of a mixing experiment demonstrated that both proteins need to be expressed within the same cell for maximum cytotoxicity, and suggest a novel role for HevCaLP. Binding assays showed that the toxin-receptor interaction is specific. Our findings confirm that HevABCC2 is the central target in Cry1A toxin mode of action, and that HevCaLP plays a supporting role in increasing Cry1A toxicity.

Author Summary

The bacterium *Bacillus thuringiensis* (Bt) produces crystal toxin proteins which are widely used as insecticides, for example in spray formulations and in transgenic crops. During the last years, first field-evolved resistance was discovered, yet the Bt toxin mode of action is not fully understood. It is known that the toxin is proteolytically activated in the insect gut prior to its interaction with protein receptors in the gut epithelium, and eventually forming membrane pores which lyse and kill midgut cells. By stably expressing two of these receptors we found that one is essential for pore formation and the other plays a novel supporting role by enhancing the mode of action. By performing a binding assay we show that the receptor-toxin-interaction is specific. A toxicity bioassay with larvae confirmed our results. This new information can be applied in designing strategies to combat the increasing incidence pest resistance to Bt toxins, and to preserve the utility of this safe and effective means of pest control.

Keywords

ABC transporter, *Bacillus thuringiensis*, Cry1A toxin, Cadherin-like, *Heliothis virescens*, Insecticide resistance

Abbreviations

ABC: ATP-binding cassette transporter; Bt: *Bacillus thuringiensis*, Hev: *Heliothis virescens*, GOI: gene of interest, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RPS18: ribosomal protein S18; Sf9 cells: cell line derived from *Spodoptera frugiperda*; wt: wild type

Introduction

Bacillus thuringiensis (Bt) is a gram-positive bacterium that produces δ-endotoxins (Cry toxins), which show high insecticidal activity [126]. The introduction of transgenic plants expressing insecticidal proteins derived from Bt has enabled a substantial reduction in the use of chemical insecticides [171]. However, it has also increased the selection pressure for Bt resistance in target species [168]. So far, field-evolved resistance to Bt sprays or transgenic crops has been observed in several pest species [168], such as *Plutella xylostella*, *Trichoplusia ni*, *Busseola fusca*, *Diabrotica virgifera*, *Spodoptera frugiperda*, and *Pectinophora gossypiella*. [131, 162-166]

The mode of action of Cry1A toxins, which are highly active against Lepidoptera, proceeds through several steps. The protoxin is ingested by the insect and solubilized in the alkaline midgut, leading to proteolytic cleavage and finally yielding a 65 kDa active toxin.[148] In the sequential binding model, Cry1A toxin monomers bind to a midgut protein with 12 cadherin domains.[150, 172] Following this, the Domain I helix α1 of the Cry1A monomer is cleaved off and the toxin monomers eventually oligomerize. The oligomer binds to other membrane-bound proteins, and finally will be irreversibly inserted into the membrane, where it forms lytic pores resulting in lethal cytosol leakage.[148] An alternative model proposes the activation of an Mg^{2+}-dependent signaling pathway subsequent to the binding of the Cry toxin to the cadherin protein, leading to necrotic cell death.[161, 173] The relationship between these models is still not fully understood.[143]

The physiological function of the 12-cadherin-domain protein is still unknown, although other members of this gene family take part in cell adhesion.[174] The absence of this

cadherin (known as HevCaLP in *Heliothis virescens*, Bt-R_1 in *Manduca sexta* and Bt-R_{175} in *Bombyx mori*) has been genetically linked to high levels of Cry1Ac resistance in the cotton pests *H. virescens*, *P. gossypiella*, and *Helicoverpa armigera*.[145, 175, 176] In addition, alkaline phosphatase (ALP) and aminopeptidase N (APN) have been proposed to function as receptors for Cry1A toxins.[146, 177, 178] The toxin oligomer shows an especially high affinity towards these proteins.[147]

Another group of proteins likely to be receptors for Cry toxins are ATP-binding cassette (ABC) transporters. This family of transmembrane proteins is associated with cell detoxification processes, such as fenvalerate resistance.[19] Although field-evolved Bt-resistance has not been observed for *Heliothis* so far, highly *Bt*-resistant strains have been selected in the laboratory.[179, 180] HevCaLP and HevABCC2 were shown to be genetically linked to Cry1Ac resistance and to the loss of Cry1Ac binding to brush border membrane vesicles (BBMVs) in these *H. virescens* strains.[156] Mutations in ABCC2 proteins also cause resistance to Cry1A toxins in *P. xylostella*, *B. mori*, and *H. armigera*; and contribute to Cry1Ac and Cry1Ca resistance in *Spodoptera exigua*.[149, 181-183]

Here we focus on investigating the detailed role of HevABCC2 in the Cry1A mode of action, as well as its potential interaction with HevCaLP. To study these mechanisms, we cloned and expressed both genes in insect cells. By using non-lytic clonal Sf9 cell lines stably expressing the proteins, we could address some questions that are difficult to approach by conventional baculovirus expression methods that ultimately lyse the cells expressing the receptors. Viability assays, binding studies, and time lapse recordings point to HevABCC2 as the central target in the Cry1A toxin mode of action and resistance in *H. virescens*, and reveal novel roles of HevCaLP in increasing Cry1A toxicity.

Results

Clonal stable cell lines expressing HevABCC2 and HevCaLP

The two putative receptors, HevABCC2 (150 kDa) and HevCaLP (195 kDa), were stably expressed in Sf9 cells after transfection and antibiotic selection (S1A Fig). Four different stable cell lines were selected: (1) untransfected wild type Sf9 cells (wt), (2) HevCaLP-expressing, (3) HevABCC2-expressing, and (4) HevABCC2- and HevCaLP-expressing cells.

Sf9 cells are a suitable expression system, since as previously shown [184] they are not susceptible to Cry1A toxins (Fig 1) and moreover they do not express endogenous putative Cry1A receptors (S1 Fig B-F). Reverse-transcription quantitative PCR (RT-qPCR) on all four cell lines revealed the absence of transcripts corresponding to *S. frugiperda* homologs of HevCaLP and HevABCC2, as well as aminopeptidase Ns (APNs) and alkaline phosphatases (ALPs). More importantly, these genes were not upregulated during the course of selection (S1 Fig C-F). All genes were significantly different expressed than the house keeping gene RPS18.

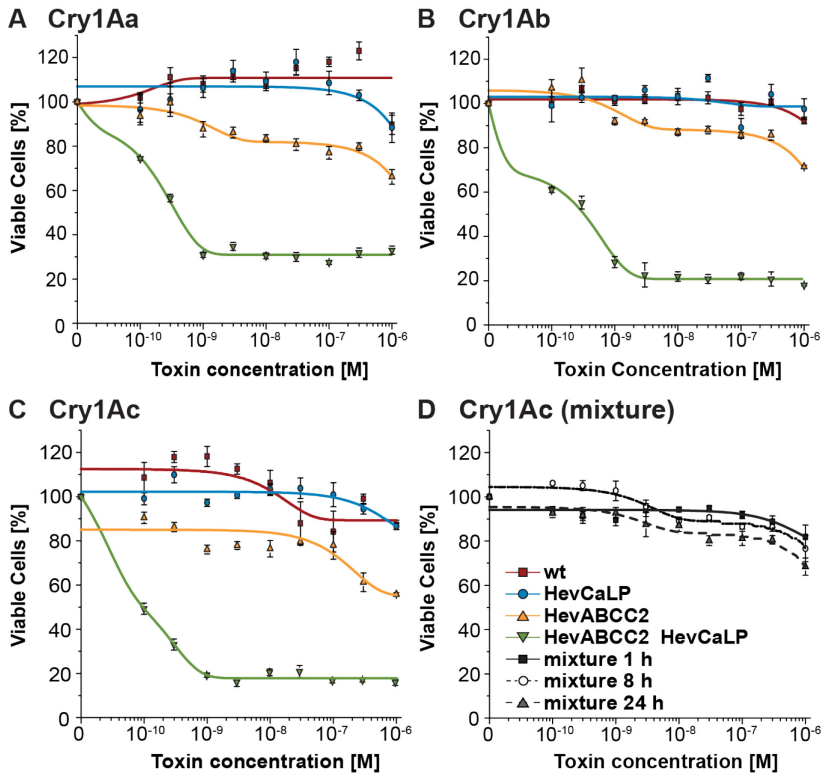


Fig 1. Effect of Cry1A toxins on cell viability (± SEM). Cry1Aa (A), Cry1Ab (B) or Cry1Ac (C) were used in concentrations 0.1 nM up to 1 µM. Red squares: untransfected Sf9 cells; blue circles: HevCaLP; yellow triangles: HevABCC2; green triangles: HevABCC2 and HevCaLP. Cells were incubated for 24 h. (D) Effect of Cry1Ac on cell viability of a one-to-one mixture of cells expressing HevABCC2 or HevCaLP. Cells were treated for 1 h (squares), 8 h (circles) or 24 h (triangles) with Cry1Ac (0.1 nM up to 1 µM). The data are based on a MTT assay (N=6). Values over 100% are due to increase in cell number due to cell division over time in the controls.

Cry1A Toxins Cause High Mortality in Doubly Transfected Cells

Sf9 cells showed high sensitivity towards Cry1C, as has been shown before.[185] A mortality rate of 96% was measured after 24 h incubation, alongside detection of granule formation within the cells and cell lysis after 4 h incubation (S2 Fig).

Treatment with Cry1Ac revealed a time and concentration dependent decrease of viability of cells expressing HevABCC2 and HevCaLP as early as 8 h of incubation ($P < 0.05$, Fig 1 and S3 Fig). HevABCC2-only expressing cells showed a trend towards decreasing viability after 24 h of incubation. No significant effect could be detected for HevCaLP-expressing cells in comparison with wt Sf9 cells. A one-to-one mixture of cells expressing HevABCC2 with cells expressing HevCaLP showed significant differences compared to cotransfected cells, but not to HevABCC2- and HevCaLP-only expressing cells (Fig 1). No effect on cell viability was observed for the control treatment with Na_2CO_3, the buffer used for Cry1A toxin solubilization (S3 Fig). Cry1Aa and Cry1Ab caused similar effects as Cry1Ac in all cell lines (Fig 1, S4 and S5 Fig). The LC50 values for the 24 h treatment were similar for all three Cry1A toxins (Tab. 1). For the one-to-one mixture of cells treated with Cry1Ac the LC50 was even higher than for wt Sf9 cells.

Table 1. LC50 values for cell lines after 24 h incubation.

Cell line	Cry1Aa [M]	Cry1Ab [M]	Cry1Ac [M]
wt	1.46×10^{-06}	1.67×10^{-06}	3.88×10^{-05}
HevCaLP	1.42×10^{-06}	5.92	6.22×10^{-06}
HevABCC2	2.45×10^{-06}	5.10×10^{-05}	2.21×10^{-05}
HevABCC2 HevCaLP	5.09×10^{-10}	4.42×10^{-10}	6.71×10^{-12}
mixture	-	-	1.19×10^{-04}

wt: untransfected Sf9 cells; Mixture: one-to-one mixture of HevCaLP- and HevABCC2 expressing cells

Cry1A toxins induce rapid cell swelling and lysis in doubly transfected cells

Time lapse recordings of doubly transfected cells treated with 10 nM Cry1A toxins showed morphological changes such as swelling, granule formation and lysis (Fig 2 and S6 Fig, S1-S4 Video). For HevABCC2-expressing cells, swelling was observed as well even though no mortality could be detected (Fig 1 and 2). No morphological changes

were observed for wt Sf9 cells. Cells expressing HevCaLP, as well as for the mixture of cells, swelling was only observed for a few cells.

After 4 h incubation, 0% of wt cells, 14% of HevCaLP-expressing cells, 87% of HevABCC2-expressing cells, and 100% of doubly transfected cells were swollen. In comparison, only 33% cells showed swelling for the mixture of cells (Fig 2). The time course of cell swelling revealed that doubly transfected cells swell faster and stronger than cells just expressing HevABCC2 (Fig 3). For HevCaLP-expressing as well as wt Sf9 cells hardly any size changes were detected. Cells of the mixture experiments showed slower cell swelling; however they reached about the same size in percentages as did HevABCC2-only expressing cells.

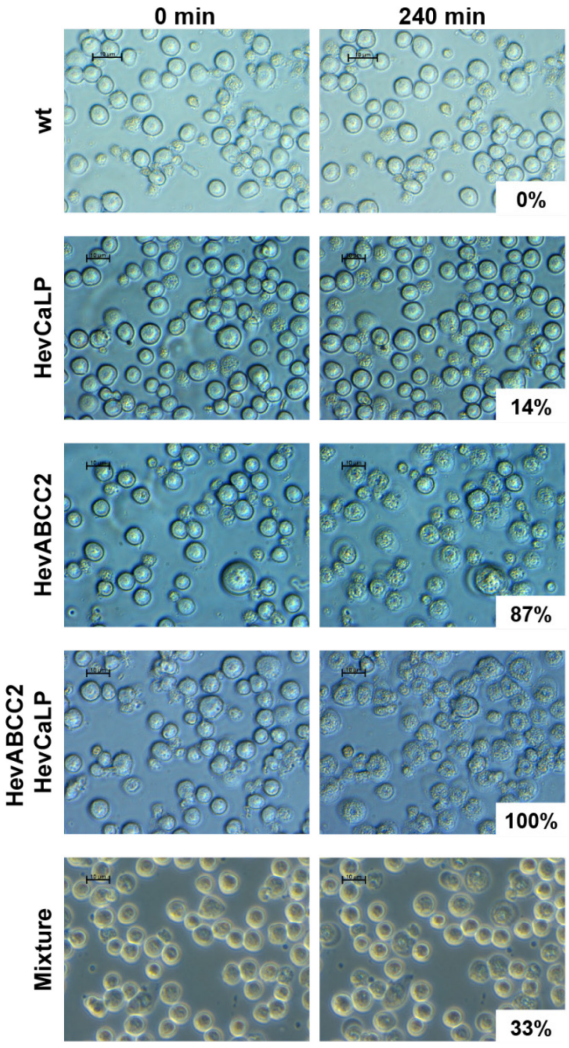


Fig 2. Morphological changes of Sf9 cells to 10 nM Cry1Ac. Cells were observed for four consecutive hours (for movies see: S1-4 Videos online). Percentage: overall percentage of swelling cells after 4 h; Scale bars: 10 µm; wt: untransfected Sf9 cells.

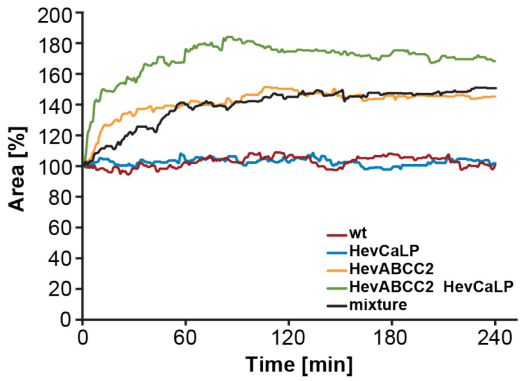


Fig 3. Time course of cell swelling. Area of cells relative to the time point 0 min. red: untransfected Sf9 cells; blue: HevCaLP; yellow: HevABCC2; green: HevABCC2 and HevCaLP; black: one-to-one mixture of cells expressing HevABCC2 or HevCaLP.

Receptor-toxin interaction reveals specific binding to HevABCC2

Binding of Cry1Ac to doubly transfected cells was detected at concentrations as low as 1 nM (Fig 4A), and there was an increase of bound toxin correlating with increasing Cry1Ac concentrations. The binding to HevABCC2-expressing cells was detected at the 1 nM concentration as well, however at a lower intensity compared to doubly transfected cells. Nonspecific binding was detected for untransfected Sf9 cells and HevCaLP-expressing cells incubated with high concentrations of Cry1Ac.

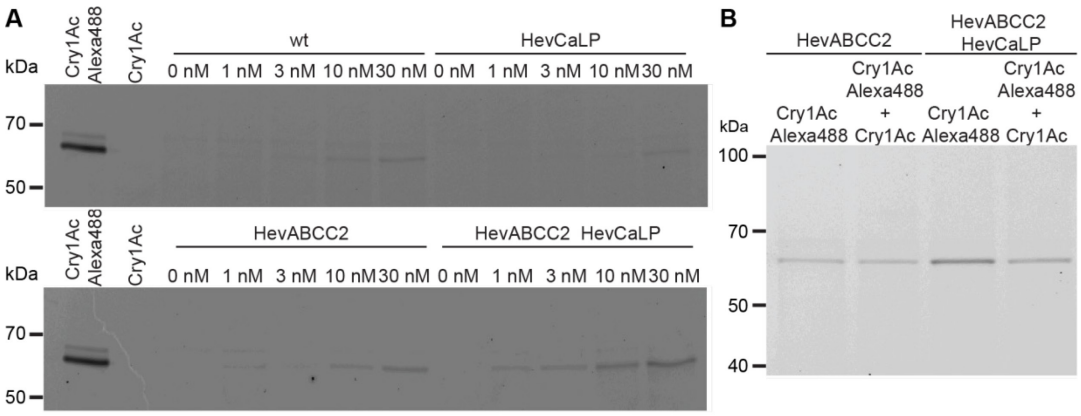


Fig 4. Binding of Cry1A toxins to Sf9 cellular membranes. A) Binding of Cry1Ac labeled with Alexa488 (1 nM up to 30 nM) to 30 µg of total cellular protein. B) Specific binding of Cry1Ac. Thirty micrograms of total cellular protein was incubated with 5 nM Cry1Ac labeled with Alexa488 and a 20-times excess of unlabeled Cry1Ac. wt: untransfected Sf9 cells.

The addition of a 20-times excess of unlabeled Cry1Ac to cells expressing HevABCC2 and to cells expressing both proteins, revealed that the binding is specific (Fig 4B). The detected fluorescence was lower for both cell lines due to the competitive binding of labeled- and unlabeled Cry1Ac.

Larvae lacking HevABCC2 and HevCaLP are resistant to three Cry1A toxins

Heliothis virescens neonate larvae of four different strains (JEN2: wt; YFO: no HevCaLP; YEE: no HevABCC2; YHD3: no HevABCC2 and no HevCaLP [156]) were fed on artificial diet supplemented with Cry1A toxins for 7 days. The JEN2 strain showed up to 40% mortality already at 0.1 µg/g diet for all three Cry1A toxins (Fig 5). The YFO and YEE neonates were able to cope with up to 1 µg/g diet. The YHD3, which has been shown before to be highly resistant to Cry1Ac [156], showed high resistance against all three Cry1A toxins on concentrations up to 100 µg/g diet. Intriguingly, all three Cry1A toxins caused similar results in all four strains, irrespective of the genetic background of the insects.

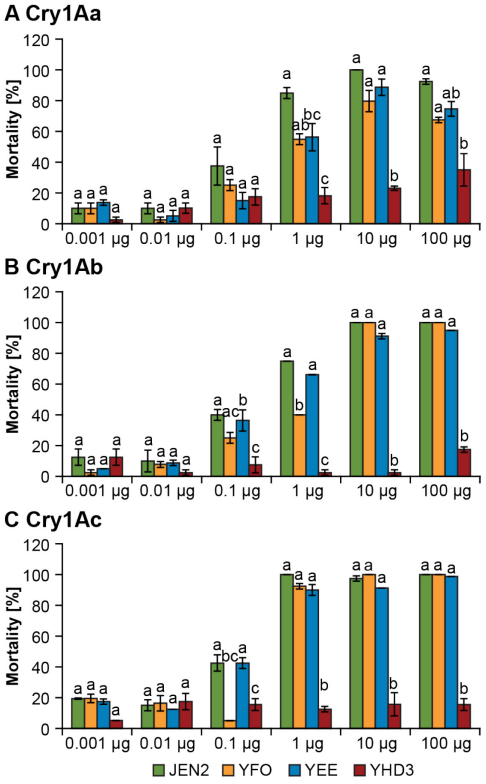


Fig 5. Mortality of *H. virescens* neonates. Bars refer to the mean mortality ± SEM after seven days. Cry1A toxins were incorporated into artificial diet. green: JEN2; yellow: YFO; blue: YEE; red: YHD3 (Colors correspond to cell lines in Fig 1 and 3); Different letters indicate significant differences (ANOVA; P-values < 0.05); N=40.

Discussion

Field-evolved resistance of insect pests to *Bacillus thuringiensis* toxins has been a continuous threat to agriculture for the last decade, albeit also putting pressure on science to resolve the mode of action of Bt toxins.[168] The aim of this study was to elucidate the role of two important receptors in the mode of action of Cry1A intoxication. By viability assays, time lapse recordings as well as binding studies, we demonstrated that HevABCC2 plays the central role in the Cry1A toxin mode of action, but also that HevCaLP is required for maximum cytotoxicity. When both proteins were expressed, up to 86% mortality was observed with severe morphological changes, such as cell swelling and lysis. The treatment with Cry1A toxins led to cell swelling in cells expressing HevABCC2 only. However, we did not observe cell swelling or mortality if only HevCaLP was stably expressed.

Corresponding results were obtained when feeding *H. virescens* neonates with different concentrations of Cry1A toxins. The YHD3 strain (lacking both HevABCC2 and HevCaLP) was significantly more resistant than the wt JEN2 strain, supporting the results obtained from the cell based assays and indicating the involvement of both proteins in Cry1A intoxication. However, the YFO strain (lacking HevCaLP) showed a higher resistance level than the YEE strain (lacking HevABCC2).

The binding assays (Fig 4) showed an increase in bound Cry1Ac for the HevABCC2-expressing cells as well as the cell line expressing both proteins. However, in wt Sf9 cells and HevCaLP-only expressing cells, binding of Cry1Ac could only be detected for high concentrations. These results confirmed that HevABCC2 is crucial for the type of binding due to irreversible insertion of the Cry1A toxin into the membrane.

The alternative signaling pathway model for Bt toxicity was proposed by Zhang et al. [173], who stably expressed *M. sexta* BT-R1 in *Trichoplusia ni* H5 cells; and observed blebbing, swelling, and death after 40 minutes of incubation with Cry1Ab. Additional experiments with inhibitors of various cell signalling pathways led to the conclusion that this mechanism of cell killing involved an adenyl cyclase/PKA signalling pathway, triggered by binding of Cry1Ab to the cadherin receptor.[173] We did not observe comparable cell blebbing, swelling, or death in Sf9 cells stably expressing HevCaLP after 240 minutes of incubation with Cry1Aa, Cry1Ab, or Cry1Ac. Therefore the same killing mechanism does not seem to be operating in our system, which to our knowledge

is the only other study with stable heterologous expression of a Cry1A-binding cadherin. Other studies, using baculovirus delivery of the entire cadherin or the membrane-proximal portion, are complicated by the eventual killing of receptor-expressing cells by the virus. The authors stated that the killing mechanism that they observed "argues against the previously postulated lytic-pore model".[173] However, the absence of an ABC protein functioning as a receptor in *T. ni* cells is a more likely explanation for the absence of pore formation and immediate cell swelling in their system, as in our HevCaLP-only-expressing Sf9 cells. The role of ABC proteins in Bt toxin mode of action was not known at the time of that study.

The synergistic yet secondary role of the cadherin was first shown by Tanaka et al. [186], who expressed the toxin-binding region (TBR) of BtR175 and BmABCC2_S from *B. mori* in Sf9 cells, separately or together using the baculovirus expression system. More toxin-induced swelling occurred in BmABCC2_S-expressing cells than in BtR175-TBR-expressing cells, but co-infected cells showed even more swelling.[186] Our results confirm this effect using proteins from *H. virescens*, but differ in the details of toxin interaction with the cadherin. Sf9 cells expressing BtR175-TBR alone showed 5% swelling with 600 nM of Cry1Ac and 20% swelling with 600 nM of Cry1Aa or Cry1Ab [186]; while we did not observe as much swelling or as much difference between the effects of different Cry1A toxins. The baculovirus expression system probably results in much higher receptor concentrations in the membrane than does our stable expression system; and amino acid sequence differences among the two species might also affect the results.

The results of the cell mixing experiment were unexpected. Under the sequential binding model, toxin monomer binding to the cadherin accelerates the additional processing step by which the α1 helix of the monomer is cleaved off.[172] These modified monomers are believed to form oligomeric pre-pore structures in solution, which are responsible for pore formation when the entire structure is inserted into the membrane. We expected that in a mixture of cells, oligomeric pre-pore structures in solution would be rapidly produced from the HevCaLP-expressing cells, and would diffuse to the HevABCC2-expressing cells for pore insertion; and therefore that the initial rate of cell swelling would be faster than in HevABCC2-expressing cells alone. However, the initial rate of cell swelling was slower in the mixture. Moreover, the amount of swelling eventually reached the same plateau as with cells expressing only HevABCC2,

suggesting that the amount of HevABCC2 present was somehow limiting the amount of swelling.

Although our experiments do not rule out a role of HevCaLP in accelerating pre-pore formation, they suggest an additional role of the toxin-binding cadherin proteins. The results with HevABCC2-only-expressing cells indicate that a limit to pore formation is eventually reached, because most cells do not continue to swell and lyse in the continuing presence of Cry1A toxins. The co-expression of HevCaLP appears to remove this limit, because cells do continue to swell and most eventually lyse. We hypothesize that HevCaLP helps to remove inserted pore structures from an association with the HevABCC2 target which is necessary for pore formation, so that the same HevABCC2 protein can catalyze the insertion of additional pre-pore structures (Fig 6). Only in cells expressing both proteins, HevCaLP can approach HevABCC2 close enough for such an interaction.

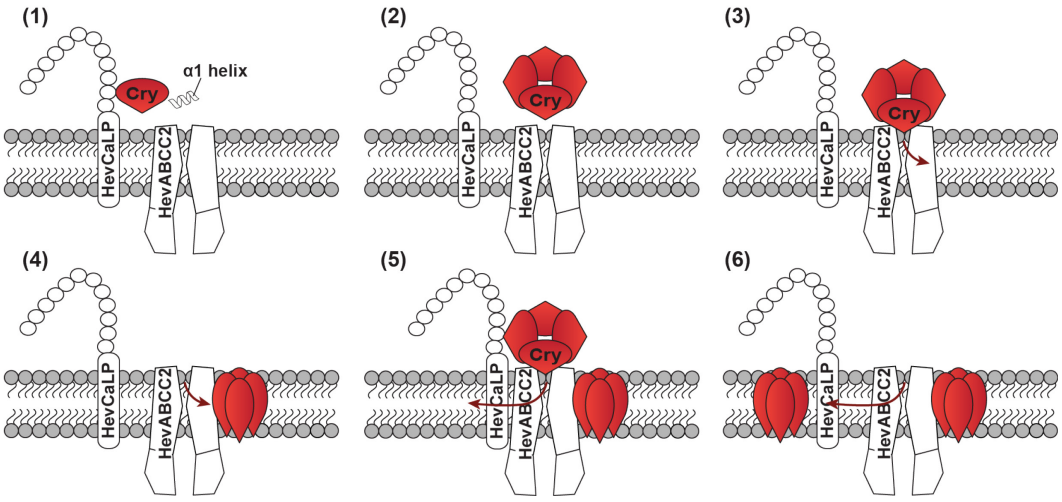


Fig 6. Proposed mode of action of Cry1A toxins. 1) Binding of a Cry1A monomer to HevCaLP and cleavage of the α1 helix. 2) Oligomerization and pre-pore formation in solution. 3) Pre-pore binding to HevABCC2. 4) Membrane insertion of the pore. 5) HevABCC2-bound pre-pore binds to HevCaLP. 6) Pre-pore cleared from HevABCC2, enhancement of pore insertion.

Other proteins known to bind Cry1A toxins, such as aminopeptidases and alkaline phosphatases [147, 148, 152, 187, 188], might also play a role in clearing ABCC2 proteins of toxin pore structures, yet this remains to be tested. We did not attempt to express APNs or ALPs from *H. virescens*. Endogenous APNs and ALPs are evidently not expressed

by the Sf9 cells; therefore Cry1A-mediated toxicity might have been even greater if these proteins were also present.

In the most successful example of developing anti-resistant strategies by using knowledge of the Bt toxin mode of action, Soberón et al designed Cry1AbMod and Cry1AcMod toxins, which lack the α1 helix and are not dependent on cadherin binding for pre-pore formation in solution.[189] These toxins are more potent on some Bt-resistant strains of Lepidoptera, including some with cadherin mutations.[190] However if the HevCaLP protein and its orthologs have the additional function that we have proposed, these modified toxins might not be completely effective in overcoming the type of Bt resistance caused by mutations that remove HevCaLP from the midgut epithelial membrane. Such mutants would also be impaired in the hypothetical clearing of the ABCC2 proteins of toxin pores, producing less cell lysis. Future investigations incorporating stable expression of other toxin-binding proteins such as ALP and APN will clarify their role in the overall mechanism of toxicity, including whether they also participate in such an ABCC2-clearing activity. Additional modifications to enhance the rate of ABCC2-clearing or to eliminate its putative dependence on HevCaLP might even be another way to design more potent and anti-resistant Bt toxins.

Materials and Methods

Sf9 cell culture maintenance

Spodoptera frugiperda derived Sf9 cells were cultured in Sf-900II serum-free medium (1x, Gibco) supplemented with 50 µg/ml Gentamicin (Invitrogen) at 27 °C. All experiments were carried out using this medium. Routine culture was performed in T75 flasks (Greiner) and cells were passaged every 3-4 days.

Generation of Sf9 clones expressing HvABCC2, HevCaLP or both proteins

Total RNA extraction from whole *H. virescens* larvae was performed using the innuPrep RNA Mini kit (analytik Jena). RNA was digested with Turbo DNAse (Ambion) and cleaned up with the RNeasy MinElute cleanup kit (Quiagen). For first-strand cDNA synthesis 900 ng RNA were used (Verso cDNA kit (Thermo Scientific).

The full-length HvABCC2 (NCBI: GQ332571) and HevCaLP (NCBI: AF367362) cDNA sequences (primers: S1 Table) were ligated between the TOPO-side of pIB/V5-His Topo TA or the restriction sides of pIZT/V5-His (HevCaLP: EcoRI- EcoRI) and

used for stable transfection of Sf9 cells. Genes were cloned in frame with the V5-epitope.

Sf9 cells were plated in 60 mm tissue culture dishes (Falcon) with approx. 70% confluency and transfected using FUGENE (Promega). Selection of cells was started 48 h post-transfection. Cloning cylinders (Sigma Aldrich) as well as limiting dilution series were applied to obtain cell clones expressing HvABCC2, HevCaLP or both proteins. Conditioned medium (the supernatant of exponentially growing Sf9 cells, 3-4 days old) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS; Gibco) was used to support cell colony growth. Culture medium contained either 500 µg/ml Zeocin (Invitrogen), 50 µg/ml Blasticidin (Invitrogen) or both antibiotics for selection. Transfection was verified by western blot and reverse-transcription quantitative PCR (RT-qPCR).

Real-time PCR

RT-qPCR reactions were carried out with three biological and two technical replicates. Total RNA extraction was performed as described above. qRT-PCR was performed with the ABsolute Blue qPCR SYBR Green Mix (Thermo Scientific), using the CFX Connect Real-time System (BioRad). Cycling conditions were 95 °C for 15 min and 40 cycles at 95 °C 15 sec, 60 °C 30 sec, 72 °C 30 sec. Primer efficiency was determined using dilution series. The resulting slope was used to calculate the efficiency as well as the amplification factor (www.thermofisher.com), which were then used in combination with the Cq-values to calculate the gene expression as copy number per 1000 molecules of a reference gene.

Furthermore, expression levels of aminopeptidase Ns (APNs) and alkaline phosphatases (ALPs) were analyzed. To evaluate the gene expression, eukaryotic initiation factor 4A **(**EiF4A) and ribosomal protein S18 (RPS18) derived from *S. frugiperda* were used as references. All primers are shown in S2 Table. Data were analyzed with an ANOVA using R.[191]

Western blotting

Cells were plated in T75 flasks. At approx. 100% confluency cells were washed and harvested in PBS. The total cellular membrane proteins were extracted (Plasma Membrane Protein Extraction Kit, abcam) and the concentration was determined using a

Bradford assay. Five micrograms of each sample were used. Samples were boiled at 94 °C (Cadherin) or 55 °C (ABC-C2) for 5 min and separated by SDS-PAGE (Criterion Precast gels, BioRad) and transferred to Immun-Blot PVDF membrane (BioRad). The blot was blocked in 1 x TBS buffer (BioRad) supplemented with 0.2% Tween 20 (Sigma Aldrich) and 5% w/v milk powder (Roth) for 1 h at room temperature. The blot was incubated with a horseradish peroxidase-conjugated (HRP-) V5 antibody overnight at 4 °C (Invitrogen). Bound antibodies were detected using an in-house detection solution (100 mM Tris pH 8.5, 90 mM coumaric acid, 250 mM luminol, 0.04% H_2O_2).

Toxin Preparation

The *Bacillus thuringiensis* strain HD73, carrying the gene for Cry1Ac, as well as two *Escherichia coli* strains harboring the genes for Cry1Aa and Cry1Ab were obtained from the *Bacillus* Genetic Stock Center (Ohio State University). Cry1A protoxins were prepared according to a modified protocol of Lee et al. [192], and were activated with trypsin at a trypsin/protoxin ratio of 1/100 (w/w) at 37 °C for 1 h. Activated toxins were further purified by anion exchange chromatography using a 1 ml RESOURCE Q column (GE Healthcare). Activated Cry1C was kindly provided by María Martínez Solís (Universitat de València, Spain).

Viability Assays and Morphological Changes

Sf9 cells were plated in 96-well cell culture plates (flat bottom, Greiner bio-one cellstar) at approx. 60% confluency. Cry1Ac (0.1 nM - 1 µM) solubilized in 50 mM Na_2CO_3 pH 9.5 was added directly to the culture medium (see above) and cells were incubated for 1 h, 8 h, or 24 h at 27 °C. The reaction volume was 100 µl. The culture medium was removed and replaced with culture medium containing 0.5 mg/ml thiazolyl blue tetrazolium blue bromide (Sigma Aldrich) to perform a MTT assay. After 2 h of incubation at 27 °C, the medium was removed and 50 µl dimethyl sulfoxide (DMSO, Sigma Aldrich) were added. Plates were put at 27 °C for 10 min. Subsequently, the 96-well plates were briefly vortexed to dissolve remaining crystals and absorbance was measured at 540 nm (Infinite m200, Tecan). A reference well, containing pure DMSO, was included and its absorbance value was afterwards subtracted from the measured absorbance of the treatments. All values were calculated in relation to untreated cells (defined as 100%). Cry1Aa and Cry1Ab concentrations from 0.1 nM - 1 µM were tested

as described for Cry1Ac. Cry1C (1 pM – 1 µM) was used as a control on the wt Sf9 cells. Two different amounts (1% and 3%) of Na_2CO_3 buffer were tested to examine effects on cell viability. Data were analyzed with an ANOVA using R.[191] LC50 values were determined in Origin8G.

For the observation of morphological changes, cells were plated in 60 mm petri dishes. After cells have attached to the bottom of the culture dish, the medium was changed to remove floating particles. Cells were incubated with 10 nM of Cry1Ac and continuously observed for 4 h on a Zeiss Axiovert200 microscope. Every 10 min a picture was taken with an AxioCam MrC5 camera and further processed with the program AxioVision AC (Release 4.3 (11-2004)).

A picture was taken every minute with a Canon EOS 600D camera. From those pictures, movies were created with VirtualDubMOD 1.5.10.3 using the following settings: framerate of 12 pictures per second, full HD 1920x1080, filter resize, filter mode Bilinear, compression Microsoft Video1.[193] Morphological changes of Cry1Aa and Cry1Ab treatment (10 nM) were observed for co-transfected cells.

The time course of cell swelling was analyzed with Fiji (ImageJ 2.0.0). The area of five randomly chosen cells was measured for each minute and the area in percentage was calculated, whereby the time point 0 min was set to be 100%. The overall percentage of swelling cells was determined for fixed time points (0 min, 60 min, 120 min, 180 min, 240 min).

Binding assays

Cry1Ac was labeled with Alexa488 according to manufacturer's instructions (molecular probes, life technologies). Cells were lysed with a hypotonic buffer (20 mM Tris pH 7.5, 0.1% Benzonase, 1% Protease Inhibitor) for 15 min at 37 °C. Samples were ground with a dounce homogenizer and three times frozen and thawed. After centrifugation, the supernatant was discarded and the pellets (crude membrane proteins) were resuspended in phosphate-buffered saline (PBS) and 1% Protease Inhibitor. Thirty micrograms crude membrane proteins were incubated with labeled Cry1Ac toxin (0 nM up to 30 nM) for 1 h at room temperature. Subsequently, samples were washed three times with cold PBS to wash off unbound Cry1Ac. Samples were boiled at 96 °C for 7 min and separated by SDS-PAGE (Criterion Precast gels, BioRad). Gels were scanned with a FUJI Film Starion FLA9000 to detect fluorescence.

To validate the specificity of the binding, a competition assay was performed. Thirty micrograms crude membrane proteins of HvABCC2-expressing cells and cells expressing both proteins were incubated with 5 nM labeled Cry1Ac and a 20-times excess of unlabeled Cry1Ac for 1 h at room temperature. Samples were washed three times with cold PBS (for SDS-Page see above).

Mortality assay with larvae

Heliothis virescens adults were mated in single pair matings, using males and females from different families to retain genetic diversity and to minimize inbreeding depression. All life stages were kept under similar conditions in an environmental chamber (55 % relative humidity (RH); 26 °C; 16 h light : 8 h dark). No special ethical approval was needed for this experiment, since *H. virescens* is an invertebrate species not underlying an ethics approval.

Neonates of four different *H. virescens* populations (JEN2: wt; YFO: no HevCaLP; YEE: no HvABCC2; YHD3: no HvABCC2 and no HevCaLP [156]) were used within one day after hatching. Pinto bean diet was prepared and cooled down [194]. Afterwards it was mixed with Cry1A toxins (concentrations 0.001 µg/g - 100 µg/g diet). The final concentration of Na_2CO_3 buffer was kept at 20% for all diets. Larvae were put individually on diet into 8-well PCR stripes with tightly closing lids. After 4 d (55 % relative humidity (RH); 26°C; 16 h light : 8 h dark), surviving larvae were big enough and a little hole was put into the lid to insure ventilation. Neonate mortality was recorded after seven days. Data were analyzed with an ANOVA using R.[191]

Acknowledgements

We thank Bianca Wurlitzer, Domenica Schnabelrauch, Antje Schmaltz, Regina Seibt, Tom Bretschneider, Franziska Zepter and Sheila Milker for technical assistance. María Martínez Solís (Universitat de València, Spain) kindly provided Cry1C. We thank Matan Shelomi for revising the manuscript. Financial support was provided by the Max-Planck-Gesellschaft.

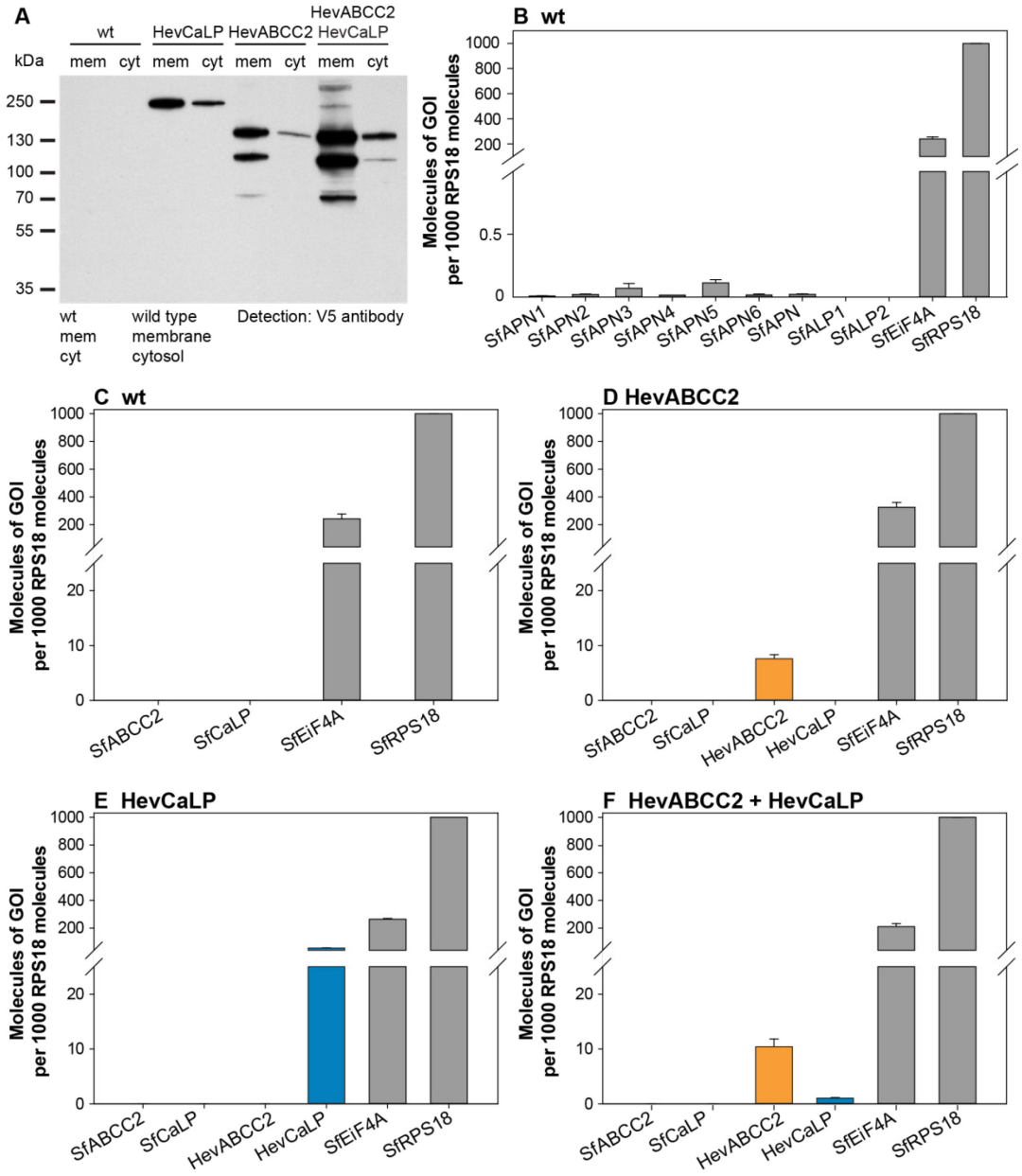
Supplementary Material

S1 Table. Primers used for amplification and cloning. Hev: *Heliothis virescens*

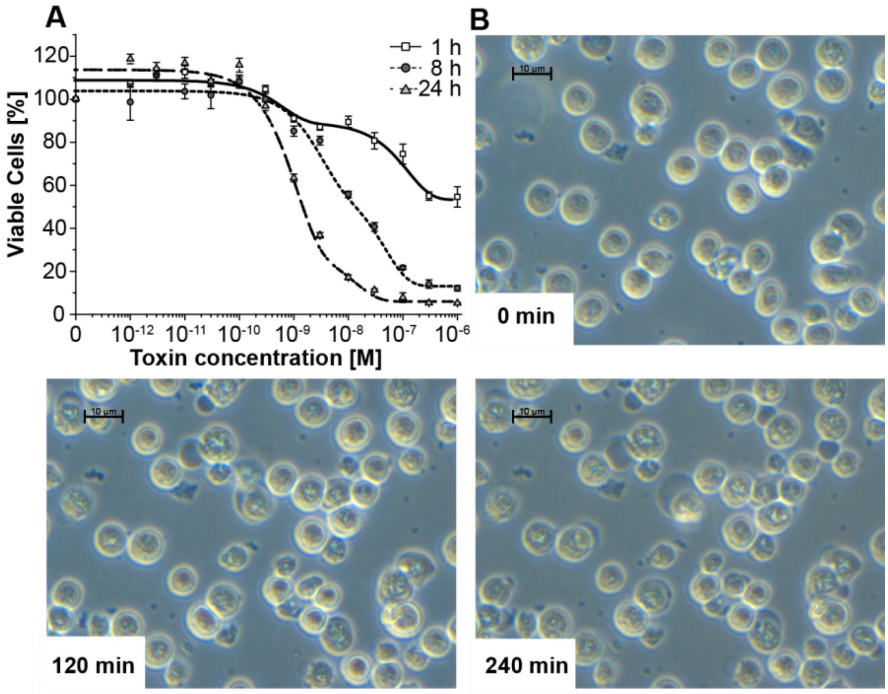
Gene	Plasmid	Primer FWD	Primer REV
HevABCC2	pIB/ V5-His Topo TA	GAG ATG GGC GTA GAA AAT AAG AAT AAT G	AGC CTC CTT ATT ATC ACT ATC GTA TTT AG
HevCaLP	pIB/ V5-His Topo TA	GAG ATG GCA GTC GAC GTG AGA ATA CTG	TCT CCT GAG CTG CGA GTT CGC
	pIZT/V5-His	ATT AGA ATT CGA GAT GGC AGT CGA CGT GAG AAT ACT GAC	ATT A GAA TTC TTT CTC CTG AGC TGC GAG TTC GCG AAC

S2 Table. Primers used for RT-qPCR. Housekeeping genes: eukaryotic initiation factor 4A **(**EiF4A) and ribosomal protein S18 (RPS18). Sf: *Spodoptera frugiperda*; Hev: *Heliothis virescens*

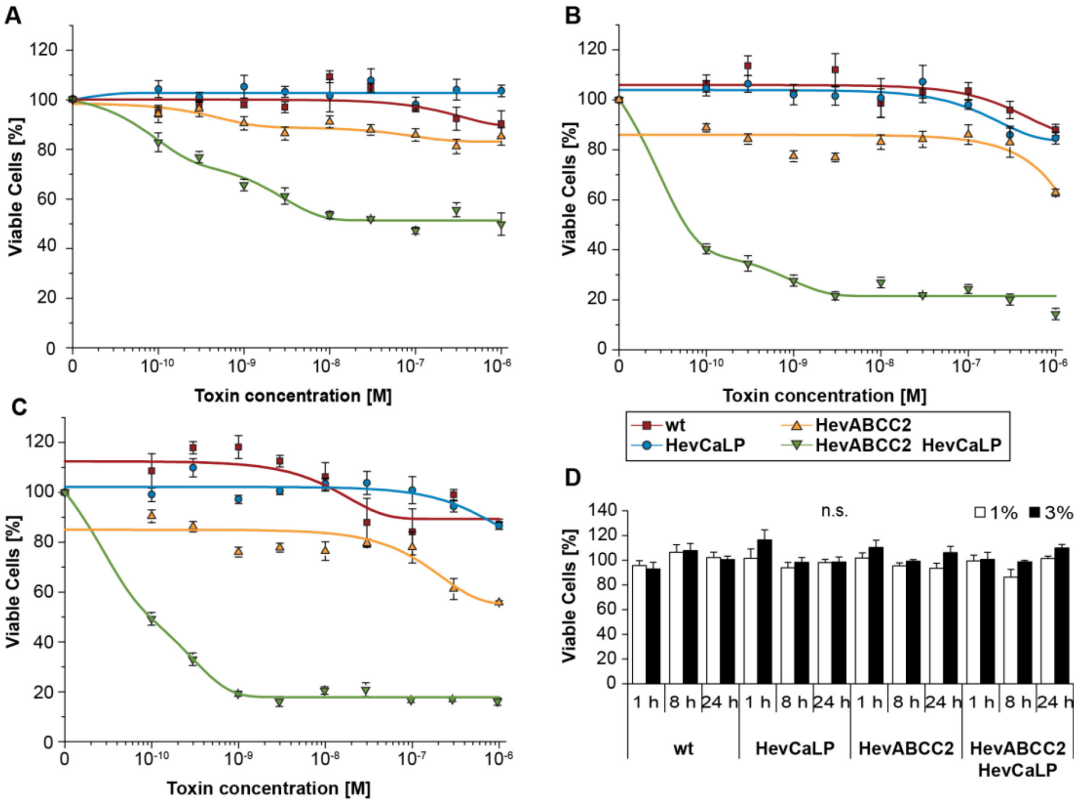
Organism	Gene	Primer FWD	Primer REV
Spodoptera frugiperda	SfABCC2	GGGACAACGTCAGCTGGTAT	CACGTGGCAAATTGTTTACG
	SfCaLP	AGACAGCGAGAGAGGAGACG	GGAATCAGAAGCCGTGACTC
	SfAPN1	CCGAGGAAGTACTGGCAGAG	CGAACACGTCATCCACAATC
	SfAPN2	ACCCAAACTGGACAAAGCTG	GTGAACATTCTCGTGGCAGA
	SfAPN3	CCCTCATCACCTCCAGCTAC	TCGTGGAACTTTGCTGGATT
	SfAPN4	ATTTCCCGGACGTCAACTTT	CCAAGTTTGGGTGGGTCTAA
	SfAPN5	CATGGCCACAGGCATTAAA	AAGCTTGTCAGCAGCATTTG
	SfAPN6	TTGTCATCCAGGCCAAAGTT	GCTTCGGTTTCATGTGGATT
	SfAPN	CTGCGGCTATTACTGGCTTC	AATGGAGCAAGTTGGAATCG
	SfALP1	GCGAGGAGGATAAACTGCAT	CCTCCGCATTCACTCCAATA
	SfALP2	CGGTCGGACTAGCTAAGACGTA	GCTATGGAGTGCACGTGGT
	SfEiF4A	ATTTACTCGCTCGTGGCATT	CCTTGAGTGCTCTCCTGTCC
	SfRPS18	AGGGTGTTGGACGCAGATAC	CTTCTGCCTGTTGAGGAACC
Heliothis virescens	HevABCC2	GTCCGGTGCTCATAACTGGT	TTTGCAACGCCTTCCATAG
	HevCaLP	GCTACCAGCGACAGTCCTTC	CAGCTCATCGTTCCAGTTGA



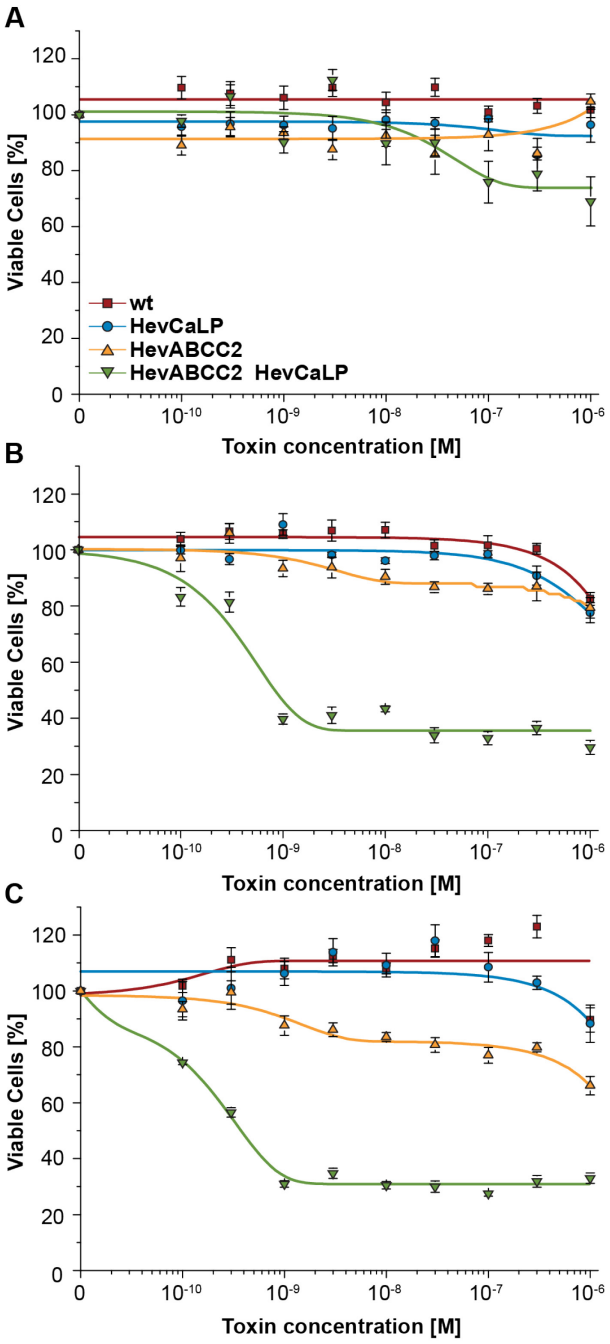
S1 Fig. Heterologous expression of HevABCC2 and HevCaLP in Sf9 cells. A) Non-lytic stable clonal Sf9 cell lines were created as follows: HevCaLP-expressing cells, HevABCC2-expressing cells, and one cell line expressing HevCaLP and HevABCC2 from *H. virescens*. The total cellular membrane proteins (mem) and the cytosol (cyt) were extracted and used for western blotting (5 µg each, detection: V5-HRP). wt: untransfected Sf9 cells. B) Detection of transcripts (RT-qPCR) of aminopeptidase N (APN) genes and alkaline phosphatase (ALP) genes in wt Sf9 cells. C-F) Detection of HevABCC2 and HevCaLP homolog transcripts (RT-qPCR) in clonal cell lines. Housekeeping genes: eukaryotic initiation factor 4A (EiF4A), ribosomal protein S18 (RPS18) derived from *S. frugiperda*. The gene expression is given as copy number per 1000 molecules RPS18 ± SEM. All primers are shown in S2 Table. Hev: *H. virescens*, Sf: *S. frugiperda*, GOI: gene of interest.



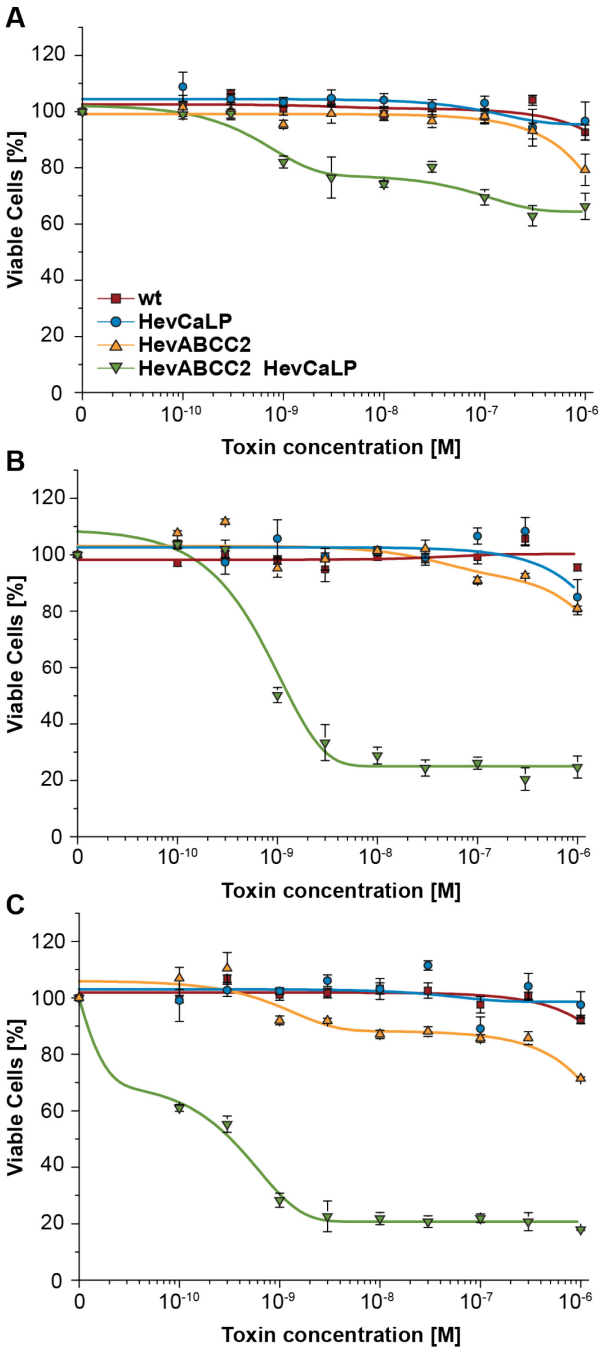
S2 Fig. Sf9 cells are susceptible to Cry1C. A) Cells were incubated for 1 h (squares), 8 h (circles) or 24 h (triangles) with Cry1C ± SEM (1 pM up to 1 µM) (N=6). B) Morphological changes upon 10 nM Cry1C treatment for four consecutive hours. Scale bars: 10 µm.



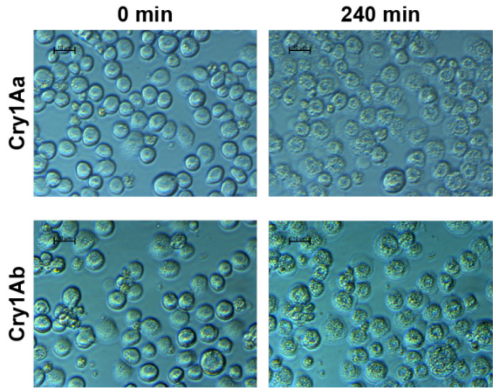
S3 Fig. Decrease of cell viability upon Cry1Ac treatment. Cells were treated with Cry1Ac (0.1 nM - 1 µM) for 1 h (A), 8 h (B) or 24 h (C). Red squares: untransfected Sf9 cells; blue circles: HevCaLP; yellow triangles: HevABCC2; green triangles: HevABCC2 and HevCaLP. Furthermore, cells were treated with Na_2CO_3 to test for an effect of the buffer, used for Cry1A toxin solubilization (D). The data are based on a MTT assay ± SEM (N=6).



S4 Fig. Viability assay of all four cell lines after treatment with Cry1Aa. Cells were treated with Cry1Aa (0.1 nM - 1 µM) for 1 h (A), 8 h (B) or 24 h (C). Cell viability was determined by a MTT assay ± SEM (N=6). Red squares: untransfected Sf9 cells; blue circles: HevCaLP; yellow triangles: HevABCC2; green triangles: HevABCC2 and HevCaLP.



S5 Fig. Effect of Cry1Ab on cell viability of clonal cell lines. Cells were treated with Cry1Ab (0.1 nM - 1 µM) for 1 h (A), 8 h (B) or 24 h (C). Cell viability was determined by a MTT assay ± SEM (N=6). Red squares: untransfected Sf9 cells; blue circles: HevCaLP; yellow triangles: HevABCC2; green triangles: HevABCC2 and HevCaLP.



S6 Fig. Susceptibility of Sf9 cells stably expressing HevABCC2 and HevCaLP to 10 nM Cry1Aa and Cry1Ab. Cells were observed for four consecutive hours. Scale bars: 10 µm

S1 Video: Transfected Sf9 cells treated with 10nM Cry1Ac for 2 h. Movies were created from individual pictures using VirtualDubMOD 1.5.10.3.

S2 Video: Cells expressing HevCaLP treated with 10nM Cry1Ac for 2 h. Movies were created from individual pictures using VirtualDubMOD 1.5.10.3.

S3 Video: Cells expressing HevABCC2 treated with 10nM Cry1Ac for 2 h. Movies were created from individual pictures using VirtualDubMOD 1.5.10.3.

S4 Video: Cells expressing HevCaLP and HevABCC2 treated with 10nM Cry1Ac for 2 h. Movies were created from individual pictures using VirtualDubMOD 1.5.10.3.

For videos refer to CD-ROM in the back of the Dissertation.

2.2 Manuscript II

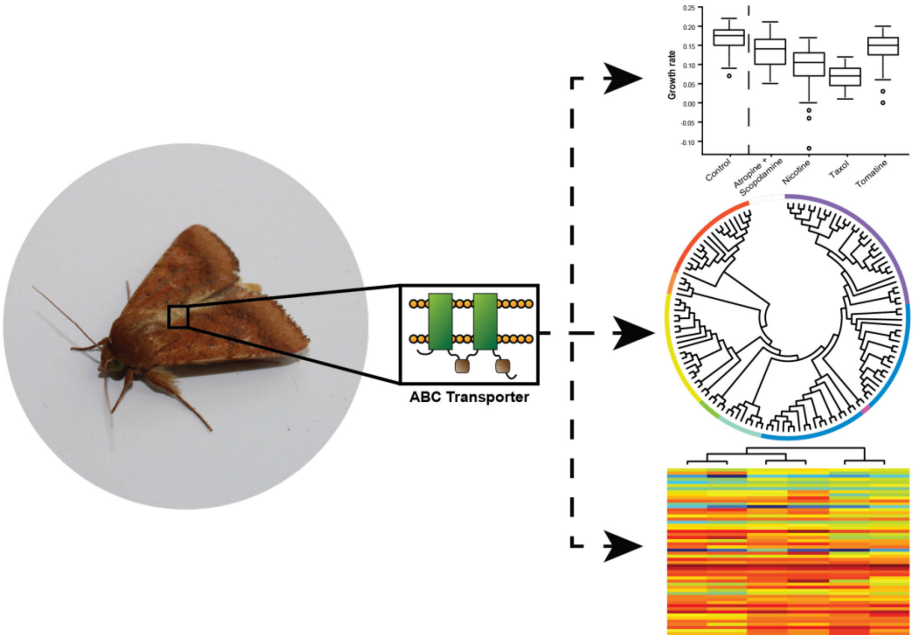
Know your ABCs: Characterization and gene expression dynamics of ABC transporters in the polyphagous herbivore *Helicoverpa armigera*

Anne Bretschneider [1], David G. Heckel [1], Heiko Vogel [1]

[1] Department of Entomology, Max-Planck Institute for Chemical Ecology, Jena 07745, Germany

Manuscript submitted on November 30th 2015 to

Insect Biochemistry and Molecular Biology



Graphical abstract

Abstract

Polyphagous insect herbivores are adapted to many different secondary metabolites of their host plants. However, little is known about the role of ATP-binding cassette (ABC) transporters, a multigene family involved in detoxification processes. To study the larval response of the generalist *Helicoverpa armigera* (Lepidoptera) and the putative role of ABC transporters, we performed developmental assays on artificial diet supplemented with secondary metabolites from host plants (atropine-scopolamine, nicotine and tomatine) and non-host plants (taxol) in combination with a replicated RNAseq experiment. A maximum likelihood phylogeny identified the subfamily affiliations of the ABC sequences. Larval performance was equal on the atropine-scopolamine diet and the tomatine diet. For the latter we could identify a treatment-specific upregulation of five ABCs in the gut. No significant developmental difference was detected between larvae fed on nicotine or taxol. This was also mirrored in the upregulation of five ABCs when fed on either of the two diets. The highest number of differentially expressed genes was recorded in the gut samples in response to feeding on secondary metabolites. Our results are consistent with the expectation of a general detoxification response in a polyphagous herbivore. This is the first study to characterize the multigene family of ABC transporters and identify gene expression changes across different developmental stages and tissues, as well as the impact of secondary metabolites in the agricultural pest *Helicoverpa armigera*.

Keywords

ABC Transporters, detoxification, generalist, *Helicoverpa armigera*, herbivore, transcriptome

Abbreviations

ABC: ATP-binding cassette (transporter), MT: Malpighian tubules, G: gut, RB: rest body, NBD: nucleotide binding domain, Ha: *Helicoverpa armigera*, Hvir: *Heliothis virescens*, Hsub: *Heliothis subflexa*, Tni: *Trichoplusia ni*, Dple: *Danaus plexippus*

Introduction

Herbivorous insects face a number of different plant defense mechanisms [3], but most importantly they encounter secondary metabolites while feeding. These substances act

as repellents and antifeedants, or may even be toxic.[49, 50] Known examples are alkaloids, terpenoids and phenolics. Insects have adapted in numerous ways to these metabolites, such as by avoiding, sequestering or converting them into less toxic compounds.[50, 195, 196] Generalist insect herbivores are thought to possess a range of general detoxifying enzymes to be pre-adapted for feeding on many different host plants [87], e.g. an expansion of gene families involved in detoxification.[197]

The cotton bollworm, *Helicoverpa armigera* (Hübner), is a generalist noctuid moth, whose larval stages feed on more than 60 cultivated plants, such as cotton, tobacco, sunflower and corn.[92, 107] Many studies have focused on different life-history aspects of *H. armigera* [45, 110, 123, 198], but only a few have focused on host plant adaptation, especially the enzymes involved in the detoxification of plant secondary metabolites.[72, 109, 199] However, all of these studies focused on cytochrome P450s, UDP-glycosyltransferases, glutathione transferases and esterases.

ATP-binding cassette (ABC) transporters are transmembrane proteins, which can be found in all forms of life, including insects.[7, 200] Known functions within the cell comprise the transport of lipids, inorganic ions and especially the detoxification of xenobiotics.[8] In insects these genes have been shown to be involved in glucoside sequestration [42], the transport of eye color pigments [201] and resistance synthetic insecticides.[19, 45] ABC transporters also act as targets for *Bacillus thuringiensis* insecticidal toxins (Bt).[202] For example Bt resistance has been genetically linked to HvABCC2 in *Heliothis virescens* [156] and HaABCC2 in *H. armigera*.[181] Just recently ABCA2, a member of the subfamily A, was shown to be involved in the Bt mode of action in *H. armigera* as well.[203]

A functional transporter consists of four core domains: two membrane spanning domains (transmembrane domain, TM), each built up from six membrane spanning α-helices, alternating with two nucleotide binding domains (NBD) located on the cytosolic side (**Fig.1A**).[7] According to their structure and domain organization, these transporters are classified into different subfamilies named from A to H. Interestingly, insects were shown to possess a larger number of ABC transporters than humans (48).[18, 37] The flour beetle *Tribolium castaneum* possesses 73 [34], and 51-53 were identified in the silk moth *Bombyx mori*.[39, 40] However, the species with the largest number of transporters is the spider mite *Tetranychus urticae* (103).[35] This high number of ABC

genes might be linked to the extreme polyphagy of this species, especially since it possesses 39 ABC C transporters, a subfamily involved in multidrug resistance.[39, 204]

It has been shown before that ABC transporter gene expression differs among tissues and developmental stages as well as after xenobiotic exposure.[21, 35, 38, 205, 206] A recent study on the plastic response of the tobacco hornworm *Manduca sexta* revealed treatment- and tissue-specific expression in larvae. Koenig et al. (2015) show that especially members of subfamilies B and C, both of which are involved in detoxification and multidrug resistance, are upregulated in the gut when larvae were fed on plants.[70] Similar results were observed in *T. urticae,* where a host plant switch induced the expression of ABC transporter genes.[35] Moreover, that study revealed a developmental-stage specific expression between embryos, larvae and adults.

Here we focus on the ABC transporter expression in the polyphagous lepidopteran species *H. armigera*. RNA sequencing (RNAseq) combined with the official *Helicoverpa* Gene Set was performed with different developmental stages as well as larval tissues to investigate when and where these genes are expressed in Lepidoptera. We report developmental- and tissue-specific ABS transporter gene expression. The influence of xenobiotics on the transcriptional response of ABC transporters was assessed by feeding larvae with host and non-host plant derived secondary metabolites prior to RNA collection. Here we show that *H. armigera* larvae developed significantly slower on all diets supplemented with secondary metabolites compared to the control. Furthermore, the xenobiotics elicited complex and compound-specific changes in ABC transporter gene expression.

Materials and Methods

2.1 Insect rearing

The TWB strain of *H. armigera* (Hübner) (Lepidoptera: Noctuidae) originated from the vicinity of Toowoomba, Queensland, Australia, in January 2003 and was transferred to the Max Planck Institute for Chemical Ecology in Jena (Germany) in August 2004. Insects were reared on artificial Bio-Serv diet (General Purpose Lepidoptera). Adults were mated in single pair matings using males and females from different families to minimize inbreeding depression and retain genetic diversity. All life stages were kept

under similar conditions in an environmental chamber (55 % relative humidity (RH); 26°C; 16 h light : 8 h dark).

2.2 Feeding assay and tissue collection

Four secondary metabolites, present in host plants of *H. armigera*, were incorporated in artificial Bio-Serv diet: tomatine (0.46 mM, Santa Cruz Biotechnology), nicotine (30.8 mM, Sigma Aldrich), atropine (10.37 mM, Sigma Aldrich) and scopolamine hydrobromide trihydrate (8.24 mM, referred to as scopolamine, Sigma Aldrich). atropine and scopolamine were mixed together in one diet, since they appear in the same host plant. Furthermore, paclitaxel (9.4 µM, referred to as taxol, Enzo Life Sciences), a secondary metabolite from a non-host plant, was tested.

Freshly molted 5th instar larvae (< 240 mg, four different families) were weighed and fed on toxin-incorporated or artificial control Bio-Serv diet for three consecutive days. Afterwards, larvae were weighed and in 1x phosphate-buffered saline (PBS, BioRad) dissected into gut (G), Malpighian tubules (MT) and rest body (RB), which comprises all tissues except the gut, malpighian tubules and the head, which was removed. From the weight data we calculated the relative growth rate, since it represents the proportional increase in mass per unit time and adjusts for initial size and the nonlinear patterns of growth over time.[207] Differences in development were statistically tested using an ANOVA in R.[191]

Furthermore, insects were reared on artificial control Bio-Serv diet to obtain samples for different developmental stages: eggs, neonates, last instar larvae, pupae, male and female adults. In addition, different tissues (head, Malpighian tubules (MT), fat body, gut, salivary gland (SG) and integument) were collected from 4th instar larvae reared on artificial control Bio-Serv diet.

2.3 RNA isolation and Illumina sequencing

RNAseq experiments were carried out with RNA isolated from larvae, which had been part of the feeding assay (see above). Eight to ten larvae from one family and the same treatment were pooled for each RNA sample (biological replicate). Four replicates were created from each treatment-tissue sample, three of which were chosen for sequencing. Total RNA was extracted according to the manufacturer's instructions (innuPREP RNA

Mini Kit, analytik Jena). The RNA was checked with the RNA6000 Nano Assay (Agilent Technologies).

Library construction and sequencing was performed by the Max Planck Genome Center, Cologne, Germany (http://mpgc.mpipz.mpg.de/home/). One µg of total RNA was used for a TruSeq RNA library and mRNA enrichment was performed. The library was sequenced with an Illumina HiSeq2500 sequencer. Approximately 10 million 100 bp single-end reads per biological replicate, treatment, and for each of the tissue samples were obtained. Quality control measures, including the filtering of high-quality reads based on the score given in fastq files, removal of reads containing primer/adaptor sequences and trimming of read length, were carried out using CLC Genomics Workbench v7.1 (http://www.clcbio.com).

2.4 Gene annotation

The *H. armigera* OGS2 predicted gene set was annotated using BLAST, Gene Ontology and InterProScan searches using BLAST2GO PRO v2.6.1 (www.blast2go.de).[208] For BLASTX searches against the non-redundant NCBI protein database (NR database) up to 20 best NR hits per transcript were retained, with an E-value cut-off $\leq 10^{-1}$ and a minimum match length of 15 amino acids to obtain the best homolog also for predicted short polypeptides. Annex [209] was used to optimize the GO term identification further by crossing the three GO categories (biological process, molecular function and cellular component) to search for name similarities, GO term relationships and enzyme relationships within metabolic pathways (Kyoto Encyclopedia of Genes and Genomes).[210] The names from the gene annotation correspond to the ones shown in the RNAseq data and the phylogeny.

2.5 Digital gene expression analysis

Digital gene expression analysis was carried out by using QSeq Software (DNAStar Inc.) to remap the Illumina reads from all samples (each replicate for all samples was mapped individually) onto the reference backbone (*H. armigera* Official GeneSet2) and then counting the sequences to estimate expression levels using previously described parameters for read mapping and normalization.[89] Biases in the sequence datasets and different transcript sizes were corrected using the RPKM algorithm (reads per kilobase of transcript per million mapped reads) to obtain correct estimates for relative

expression levels. To control for the effect of global normalization using the RPKM method, we also analyzed a number of highly-conserved housekeeping genes that are used as control genes for quantitative PCR. These included several genes encoding ribosomal proteins (RpL3, RpL4, RpL13, RpL15, RpS2, RpS8, RpS12, RpS15a, RpS18 and RpS24), elongation factor 1alpha and eukaryotic translation initiation factors 4 and 5. The corresponding genes were inspected for overall expression levels across samples and treatments and were found to display expression level differences (based on RPKM values) lower than 1.3-fold between samples, indicating they were not differentially expressed and validating them as housekeeping genes.

2.6 Phylogeny

The phylogenetic analysis comprised in total 124 amino acid sequences. 54 ABC transporters were identified in the OGS2 of *H. armigera*. All sequences were cut down to contain only the nucleotide binding domain (NBD), the most conserved region among all ABC transporters. Full ABC transporters were cut between the two NBDs and both halves were separately included (**Fig. 1**). A MUSCLE Alignment was performed with the default settings using MEGA5. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Reverse Transcriptase + Freq. model with a Gamma distribution (the determined best model) combined with a bootstrap analysis (1000 replicates). All positions with 0% site coverage were eliminated. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Apart from *H. armigera*, ABC transporter sequences from *Danaus plexippus* (17 ABCs), *Heliothis virescens* (1 ABC), *Heliothis subflexa* (2 ABCs) and *Trichoplusia ni* (5 ABCs) were included.

Results

3.1 Larval development on different host- and non-host plant derived secondary metabolites

Helicoverpa armigera larvae (freshly molted 5th instar) were fed on artificial diet for three consecutive days. Larval weight data were used to calculate the relative growth rates.

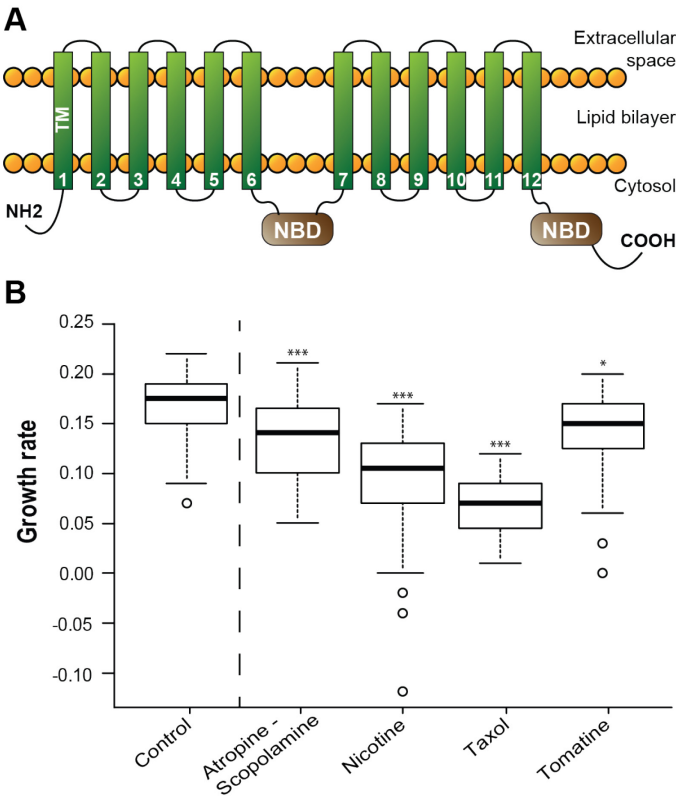


Figure 1: A) The core structure of a full ABC transporter, containing two transmembrane domains (TMs, composed of 12 membrane-spanning α-helices) alternating with two nucleotide binding domains (NBDs). B) Relative growth rate of *H. armigera* larvae on artificial diet supplemented with secondary metabolites after three consecutive days of feeding compared to control diet. nicotine 30.8 mM, tomatine 0.46 mM, taxol 9.4 µM, atropine 10.37 mM, scopolamine 8.24 mM, Tukey HSD: P-value * ≤ 0.05, * ≤ 0.001; N = 40 / diet**

Larvae fed on control diet gained significantly more weight (average growth rate 0.17) than the larvae fed on all other diets (P-value < 0.05 or < 0.001 respectively) (**Fig. 1B**). No difference was observed between larvae fed on tomatine (average growth rate 0.14) and larvae fed on atropine-scopolamine (average growth rate 0.13, P-value > 0.05, **Tab. 1**). Feeding on nicotine caused the slowest development of all of the host plant derived compounds (growth rate 0.09). Moreover, we recorded the overall slowest development when larvae were fed on artificial diet supplemented with taxol, a secondary metabolite from a non-host plant. These larvae displayed the lowest average growth rate (0.06), but we did not detect a significant difference in comparison to nicotine-fed larvae (P-value > 0.05, **Tab. 1**).

Table 1: P-values for larval development on artificial diet supplemented with secondary metabolites. Larvae were fed for three consecutive days. Tukey HSD, N = 40/diet.

	Atropine-Scopolamine	Nicotine	Taxol	Tomatine	Control
Atropine-Scopolamine	-	-	-	-	-
Nicotine	0.000	-	-	-	-
Taxol	0.000	0.172	-	-	-
Tomatine	0.971	0.000	0.000	-	-
Control	0.000	0.000	0.000	0.015	-

3.2 Phylogeny of ABC Transporters in *Helicoverpa armigera*

We constructed an overall phylogenetic tree of the multigene family of ABC transporters using 124 sequences from five different species (**Fig. 2**). The sequences formed clades according to the eight known ABC transporter subfamilies: A to H. This phylogeny was used to demonstrate the affiliation of the sequences analyzed in the RNAseq to their respective subfamilies, and it revealed the orthologous relationship between the sequences from the different lepidopteran species.

The largest family, ABCG, is composed of 16 *H. armigera* and 4 *D. plexippus* ABCs, and the smallest, ABCD, just comprises two *H. armigera* sequences. The major eye pigment precursor transporters white (ABCG4), brown (ABCG1) and scarlet (ABCG3) could be identified as well.[211] Interestingly, the N- and C-terminal halves of the ABCCs cluster on two different branches. The *H. armigera* ABCC2 clusters with the ortholog from *H. virescens,* both are targets for Bt toxins [156, 181] and ABCC3 forms a clade with the *Heliothis subflexa* ortholog. The latter is on the same branch as the ABCBs, as has been shown before.[37] The subfamily B is divided into half and full transporters. This suggests a duplication event, which caused the formation of the two subfamilies B and C and then gave rise to diversification within the two families. The subfamily ABCE is highly conserved across the species. Within the subfamily A the transporters ABCA1 and ABCA2 could be identified. The latter is involved in the Bt mode of action in *H. armigera*.[203]

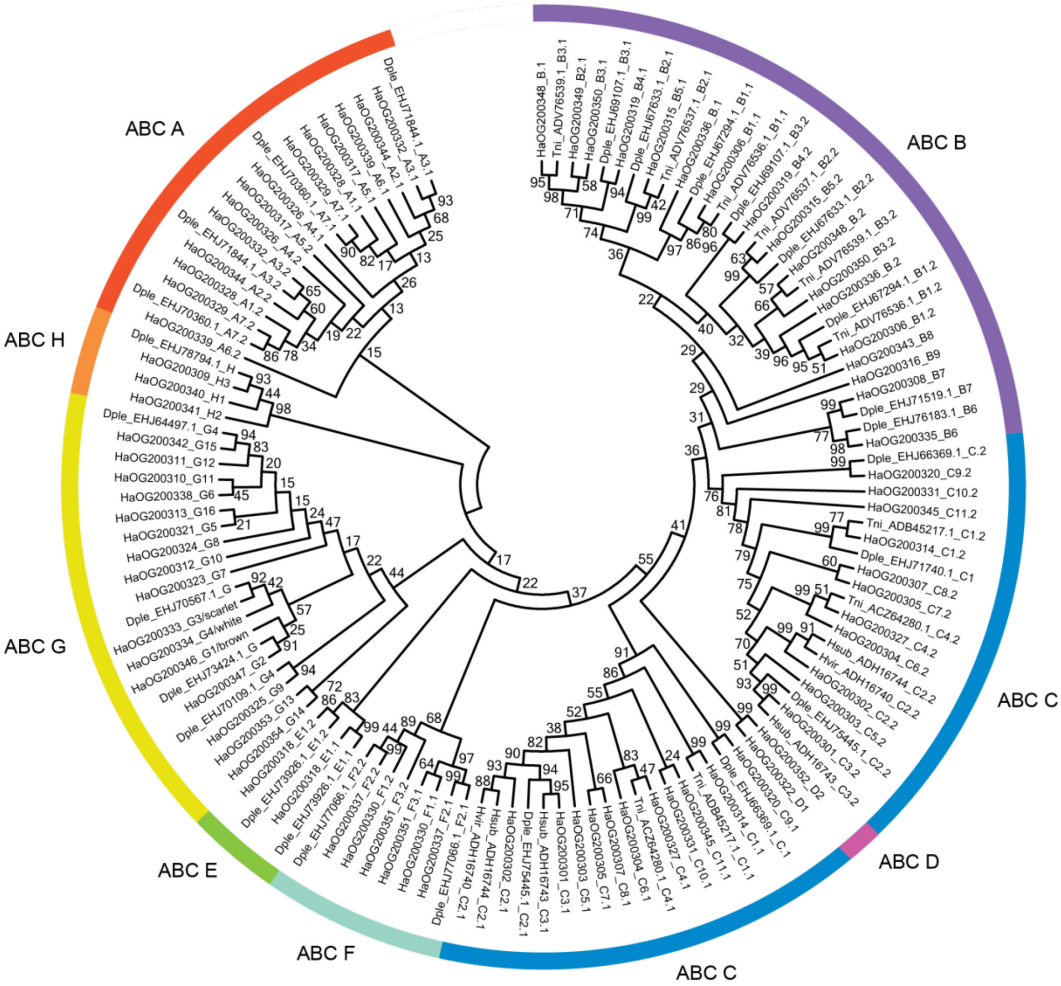


Figure 2: Phylogenetic tree of ABC Transporters in *H. armigera*. The analysis involved 124 amino acid sequences. All sequences were cut down to contain only the nucleotide binding domain (NBD). Full transporters are represented with two NBDs each. The sequences were aligned using MUSCLE. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Reverse Transcriptase + Freq. model with a Gamma distribution (Dimmic et al., 2002). A bootstrap analysis (1000 replicates) was conducted. All positions with 0% site coverage were eliminated. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The phylogenetic analysis was conducted in MEGA5. *Ha*, *Helicoverpa armigera*; *Dple*, *Danaus plexippus*; *Hvir*, *Heliothis virescens*; *Hsub*, *Heliothis subflexa*; *Tni*, *Trichoplusia ni*

3.3 ABC transporter expression across developmental stages

Initially, we were interested to determine when ABC transporters are expressed throughout insect development. For this reason, RNA was extracted from different developmental stages and sequenced. We mapped the sequencing reads obtained from the individual samples to the 17098 genes in the official gene set of the generalist *H. armigera*. **Fig. 3A** shows the log2-transformed expression values (RPKM) for the 54

ABC transporters, which were identified in the genome. We performed hierarchical clustering using a Euclidean distance metric and the centroid linkage method. Remarkably, the developmental stages cluster chronologically. The larval stages and the adult stages cluster together. Surprisingly, the pupal stage is more similar to the adult life stages regarding its transcription profile than to the larval stages. The eggs cluster away from all the other samples.

Some ABC transporters (ABCE1, ABCF2 and ABCG12) are generally highly expressed throughout all of the developmental stages, whereas others show developmental specific expression patterns. For example ABCB9, ABCG16 and members of the C subfamily (ABCC1, ABCC2, ABCC7 and ABCC9) were most highly expressed in the eggs. Two of those (ABCC1, ABCC2) are also upregulated in the larvae, alongside of ABCG4. Female and male adults were quite similar regarding the expression profile of ABCs. Remarkably, ABCC8 is massively upregulated in adults and pupa (fold-change > 139 when compared to eggs), though its overall expression is low. Proteins of the E and F subfamily mainly regulate protein synthesis and expression. Members of these two subfamilies are equally expressed throughout all developmental stages.

3.4 ABC transporter expression in larval tissues

In order to identify potential tissue-specific ABC transporter genes, we analyzed their expression in different larval tissues, in order to determine where those genes are expressed. Fourth instar larvae were fed on artificial control diet and afterwards dissected into different tissues. Contrary to our expectation, when comparing ABC transporter gene expression profiles, the gut and the Malpighian tubule samples do not cluster together (**Fig. 3B**). Since both tissues are involved in detoxification and excretion mechanisms, it was expected that they express ABCs similarly. Instead the gut clusters together with the fat body and the Malpighian tubules form a branch together with the integument. Furthermore, the head and the salivary gland cluster on the same branch, away from the other samples.

As has been observed for the developmental stages, some transporters (ABCE1, ABCF2 and ABCG12) are equally expressed throughout all of the tissues, whereas others show tissue-specific expression patterns. First of all, we detected no ABC transporter exclusively expressed in the integument and only a single transporter (ABCG13) that was highest expressed in the salivary gland. In the gut, which is the main excretory

organ of a larva, only three genes were detected with higher transcript levels (ABCA7, ABCB1 and ABCC6) (fold change > 6 compared to the head).

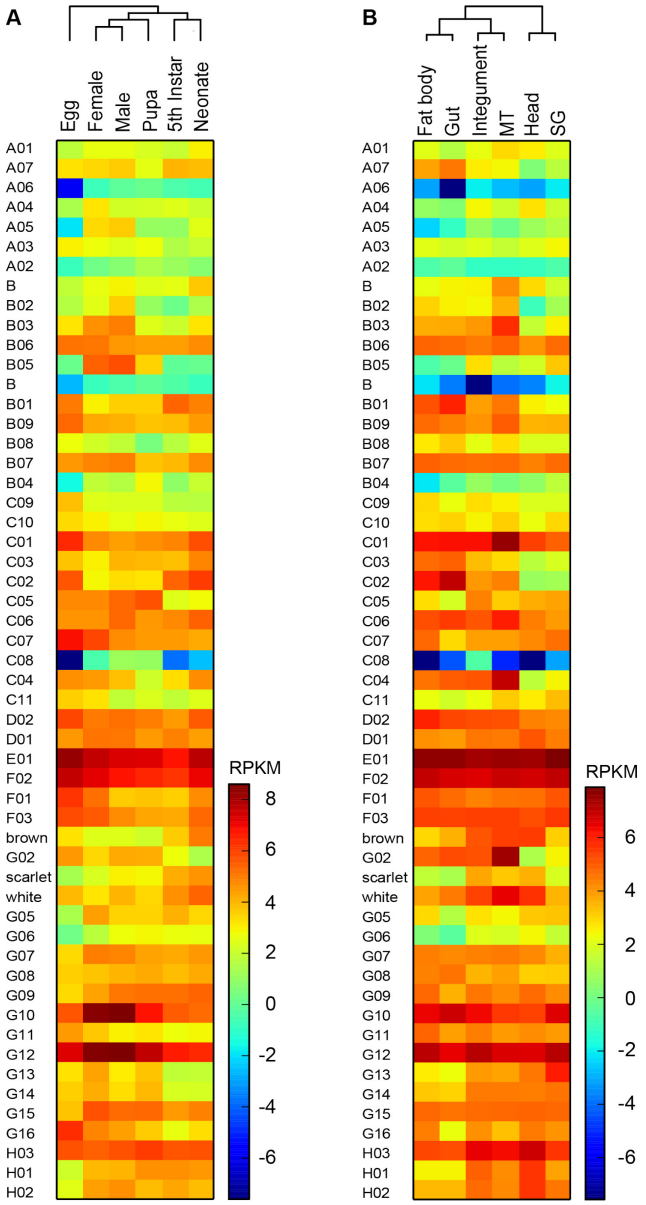


Figure 3: Gene expression of ABC transporters in *H. armigera* throughout development (A) and 4th instar tissues (B). Values are based on log2-transformed RPKM values relative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). MT, Malpighian tubules; SG, salivary gland

These genes are further upregulated when larvae are fed on secondary metabolites (see below). As expected, members of the subfamilies B and C are highest expressed in the Malpighian tubules (ABCB, ABCB2 + ABCB3, ABCC1, ABCC4, ABCC6, as well as ABCG2 and ABCG4). Since these subfamilies are known to be involved in the detoxification of xenobiotics [39], it is reasonable that they are expressed in a excretory

organ. Interestingly, the transporters of the subfamily H are highest expressed in the head of 4th instar larvae (**Fig. 3B**). So far, no phenotype or function has been associated with this subfamily [17], yet similar results were obtained by Koenig et al.[70]

3.5 Influence of secondary metabolites on ABC transporter expression

Next we investigated how transporter gene expression is influenced by xenobiotics. We fed 5th instar larvae with artificial diet supplemented with secondary metabolites and compared ABC transporter gene expression to larvae fed on artificial control diet (**Fig. 4**).

There is a clear division between the three tissues (MT, G and RB). The control samples always cluster away from the treatments. However, it is noticeable that the G-control sample is on its own branch. We identified significant ABC transporter upregulation in all three tissues, yet the strongest response was detected in the gut (MT: 12 genes, G: 53 genes, RB: 23 genes) (**Tab. 2**). We also detected treatment- and tissue-specific transcriptional upregulation of a number of ABC genes. For example taxol treatment induced the expression of ABCE1 in the Malpighian tubules compared to control samples. This was the only significant response detected for the ABCE1 gene. In addition, the upregulation of ABCG11 in MT and G samples was exclusively elicited by nicotine feeding. This alkaloid also induced the expression of ABCC8 but expression changes were restricted to the gut of nicotine-treated larvae. ABCB, ABCB2, ABCC1, ABCC11 and ABCG2 showed a significant, treatment specific (tomatine) upregulation in gut samples compared to control samples. This might be a sign that they are directly involved in the detoxification of tomatine. Moreover, we detected tissue-specific upregulations in the rest body. ABCD2 was 5-fold upregulated in RB samples of nicotine fed insects (P-value 0.028). The ABCD subfamily is involved in the transport of long chained-fatty acids.[25] This was the only response that could be detected for this gene. Surprisingly, the feeding on atropine-scopolamine elicits only one treatment- as well as tissue-specific transcriptional response (ABCG13 in the rest body). ABCA4, ABCB9, ABCC4 and ABCC7 were significantly different expressed in the gut and ABCA7, ABCB3 and ABCG10 in the rest body across all treatments compared to the control. This indicates a rather unspecific defense mechanism. Despite that, this gene is

overall very lowly expressed compared to the other genes. ABCB5 shows a 23-fold upregulation in gut samples of larvae fed on nicotine (P-value 0.011).

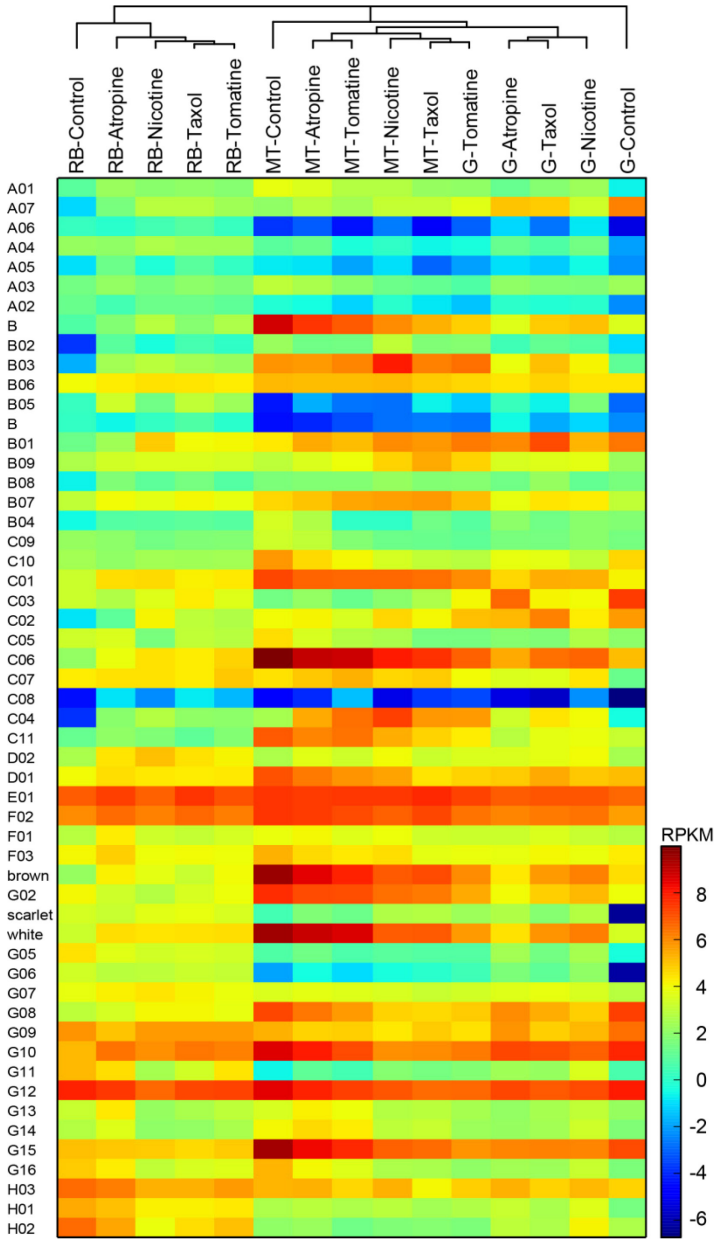


Figure 4: Gene expression of ABC transporters in *H. armigera* larvae fed on artificial diet supplemented with secondary metabolites (see Fig 1). Values are based on log2-transformed RPKM values relative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). G, gut; MT, Malpighian tubules; RB, rest body

The treatments with nicotine or taxol provoked similar effects. In addition to resulting in slowest larval development, they led to the upregulation of ABCG3/scarlet in the Malpighian tubules and ABCB1, ABCC2 and ABCC6 in the rest body. This could indicate that the cellular responses to cope with nicotine and taxol are similar.

Interestingly, one transporter was identified to be upregulated in all three tissues when compared to the control samples: ABCB3 showed up to 43-fold in tomatine gut samples. ABCB3, also known as TAP 2, is important for antigen processing in mammals.[8] The function in insects is not fully understood and thus it might have an important role for coping with plant secondary metabolites.[70]

Table 2: Differential gene expression in tissues of larvae fed on artificial diet supplemented with secondary metabolites compared to control diet. Significant upregulation (P-value < 0.05)

Treatment	Total	MT	Gut	Rest body
Atropine-Scopolamine	25	0	10	5
Nicotine	58	5	17	8
Taxol	50	5	9	7
Tomatine	44	2	17	3

The gut and the Malpighian tubules showed some overlap regarding their expression profile. The member 7 of subfamily B is significantly upregulated in nicotine, taxol and tomatine fed insects. The ABC transporter G1/brown displayed upregulation in gut (tomatine) and rest body samples (atropine-scopolamine and nicotine). ABCG1/brown is associated with cholesterol transport in vertebrates and the transport of eye pigments in *Drosophila melanogaster*.[43, 212]

Overall, the feeding on nicotine and taxol supplemented diet had a very strong influence on the ABC transporter gene expression in comparison to the control and the two other treatments (**Tab. 2**). This is reflected in the growth rate (**Fig. 1B**).

Discussion

This study was conducted to investigate the larval development of *Helicoverpa armigera* on different host- and non-host plant derived secondary metabolites with a special focus on the transcriptional response of ABC transporters, potentially involved in detoxification processes. To address this, a developmental assay was combined with a replicated RNAseq approach. *Helicoverpa* larvae were fed on different diets supplemented with secondary metabolites. Furthermore, the transcriptional plasticity of ABC transporters was addressed across different developmental stages as well as larval tissues.

The developmental data show that *Helicoverpa* is able to develop in the presence of these five secondary metabolites, even though larvae developed significantly lower on all diets compared to the control. Atropine-scopolamine, nicotine, tomatine and taxol cause a delayed development in larvae. In the case of the latter it might even be a toxic effect, since larvae showed the lowest growth rate. Atropine-scopolamine showed no significant difference to tomatine regarding the larval growth rate. These finding indicate that these three alkaloids challenge the insect system in similar ways.

To uncover the mechanisms underlying these phenotypic differences, we analyzed the transcriptional changes of ABC transporters in the larval gut and Malpighian tubules, the two most important organs for detoxification. The lowest growth rates correlate with the highest number of differentially expressed genes (see nicotine and taxol, **Fig. 1** and **Tab. 2**). This indicates that the larvae were under cell or dietary stress when fed on nicotine and taxol. Of all the secondary metabolites which are present in *Helicoverpa* host plants and were used in our assays, nicotine feeding resulted in the lowest larval growth rate. We identified compound specific transcriptional signatures in all tissues of nicotine fed larvae. These results suggest that the generalist *Helicoverpa* actively invests into nicotine detoxification mechanisms at the expense of slower larval development. ABCB3 was found to be induced in *Manduca* larvae fed on *Nicotiana attenuata* [70], and in Malpighian tubules of *Heliocverpa* larvae fed on nicotine-containing diet. However, ABCB3 is also upregulated in the gut when *Helicoverpa* larvae were fed other secondary metabolites. This suggests that ABCB3 has a broader role in xenobiotic detoxification, either by directly transporting these substances or by being part of a detoxification pathway. However, we identified a nicotine-specific upregulation of ABCG11 in the Malpighian tubules and the gut as well, hinting to a specific detoxification of nicotine by certain ABC transporters. This had been proposed before in *M. sexta*.[83, 84] ABC transporters may also be involved in the transport of nicotine in the plant.[85]

ABC transporters were shown to be involved in taxol detoxification, a xenobiotic known for its interference with microtubules during cell division.[213, 214] This xenobiotic naturally occurs in *Taxus brevifolia*, a non-host plant of *H. armigera*. Larvae treated with taxol showed the lowest relative growth rate, but not the highest number of differentially expressed genes.

Our findings suggest that *H. armigera* has the potential to develop on taxol containing plants, since it upregulate ABCB1, ABCC2 and ABCC6 in the rest body, as well as ABCB1 in response to taxol feeding. It was shown before in vertebrates that taxol is detoxified by ABCB1.[213, 215]

Larvae fed on tomatine showed the highest growth rate, alongside of a compound-specific upregulation of five ABCs in the gut of tomatine fed insects (ABCB, ABCB2, ABCC1, ABCC11 and ABCG2). None of these genes were differentially expressed in any of the other treatments. These results could indicate that *H. armigera* has adapted to feed on tomatine-containing plants by expressing specific ABC transporters. ABCC1 (referred to as ABCC3 in *M. sexta*) and ABCC11 were identified in the mouthparts of *M. sexta* larvae when fed on host and non-host plants as well.[70] The fact that both studies independently identified ABCC11 and ABCC1, and that the latter is known for its ability to remove toxic organic ions [216], suggests the involvement of both proteins in coping with secondary metabolites. However, the specific function of ABCC1 and ABCC11 in *Helicoverpa* may only be discovered by further studies.

The ABC transporter B7 was upregulated across all tissues in taxol-fed larvae, as well as in nicotine- and tomatine-fed insects. In human cells ABCB7 is involved in iron transfer from the mitochondria to the cytosol.[217] However, in *Helicoverpa* this gene might be part of a general defense mechanism to protect the insect from toxic compounds.

Larval exposure to nicotine and taxol provoked the upregulation of the same genes in the larval gut (ABCB1, ABCC2 and ABCC6). This might indicate that, although they have different effects on *H. armigera* and only nicotine is encountered in nature, the detox mechanism for these two secondary metabolites are similar.

We observed a complex pattern of ABC transporter expression across different developmental stages of *H. armigera*. Similar results have been found in the herbivorous spider mite *T. urticae*, as well as for cytochrome P450s in the mosquito *Anopheles gambiae*.[35, 218] These results are in agreement with different diets and nutrient requirements during an insect's life span. In addition, we identified specific transcriptional signatures of ABC transporters in different larval tissues. This was also observed in the leaf beetle *Chrysomela populi*.[38] This tissue-specific expression pattern underlines the different functions of the tissues and illustrates how molecules are shuttled throughout the larvae. For example it was shown in *B. mori* that the fat body is

an important reservoir for nutrients, but also that it expresses genes involved in xenobiotic metabolism.[219] The fact that we identified ABC transporters which are expressed in the fat body, could explain how the nutrients are transported that are stored in this tissue.

Helicoverpa armigera larvae feed on a vast amount of different plants and therefore the larvae encounter a large number of defense mechanisms, such as secondary metabolites. In order to survive these compounds and maintain larval growth, a generalist herbivore is expected to possess many different biochemical defense strategies. Our results illustrate that most of the ABC transporters are upregulated in response to multiple, different secondary metabolites, rather than each displaying a distinct, compound-specific expression pattern.

In summary, this study provides insights into ABC transporter gene expression in the polyphagous agricultural pest *H. armigera* and additionally offers a basis for future studies on secondary metabolite detoxification by ABC transporters in lepidopteran species.

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2.3 Manuscript III

The plastic response of *Manduca sexta* to host and non-host plants

Christopher Koenig [1], Anne Bretschneider [2], David G. Heckel [2], Ewald Grosse-Wilde [1], Bill S. Hansson [1], Heiko Vogel [2]

[1] Department of Evolutionary Neuroethology, Max-Planck Institute for Chemical Ecology, Jena 07745, Germany
[2] Department of Entomology, Max-Planck Institute for Chemical Ecology, Jena 07745, Germany

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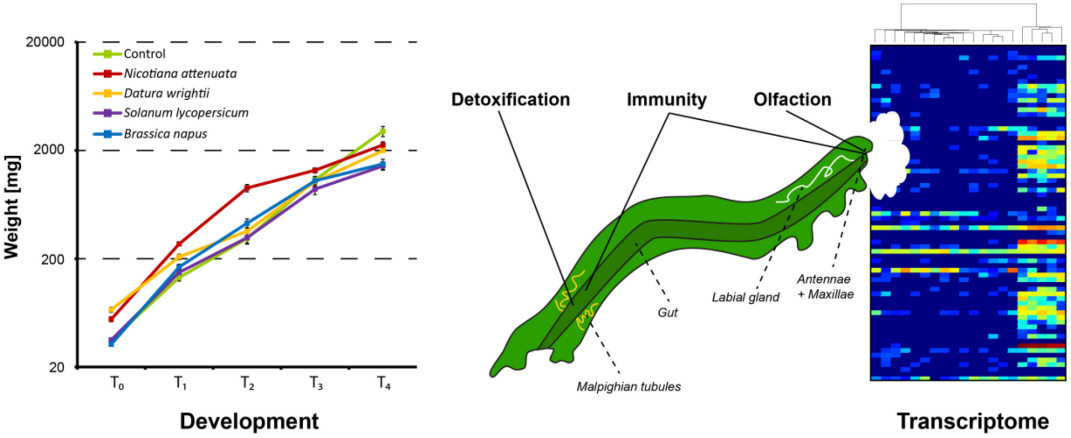

Graphical abstract

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The plastic response of *Manduca sexta* to host and non-host plants

Christopher Koenig [a, 1], Anne Bretschneider [b, 1], David G. Heckel [b], Ewald Grosse-Wilde [a], Bill S. Hansson [a], Heiko Vogel [b, *]

[a] Department of Evolutionary Neuroethology, Max-Planck Institute for Chemical Ecology, Jena 07745, Germany
[b] Department of Entomology, Max-Planck Institute for Chemical Ecology, Jena 07745, Germany

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ABSTRACT

Specialist insect herbivores have evolved efficient ways to adapt to the major defenses of their host plants. Although *Manduca sexta*, specialized on Solanaceous plants, has become a model organism for insect molecular biology, little is known about its adaptive responses to the chemical defenses of its hosts. To study larval performance and transcriptomic responses to host and non-host plants, we conducted developmental assays and replicated RNAseq experiments with *Manduca* larvae fed on different Solanaceous plants as well as on a Brassicaceous non-host plant, *Brassica napus*. *Manduca* larvae developed fastest on *Nicotiana attenuata*, but no significant differences in performance were found on larvae fed on other Solanaceae or the non-host *B. napus*. The RNAseq experiments revealed that *Manduca* larvae display plastic responses at the gene expression level, and transcriptional signatures specific to the challenges of each host- and non-host plant. Our observations are not consistent with expectations that specialist herbivores would perform poorly on non-host plants. Instead, our findings demonstrate the ability of this specialized insect herbivore to efficiently use a larger repertoire of host plants than it utilizes in the field.

1. Introduction

The tobacco hornworm (*Manduca sexta*, henceforth called *Manduca*) has become an important model system in insect science (Baldwin, 2001; Riddiford et al., 2003; Shields and Hildebrand, 2001; Späthe et al., 2013). There are many studies on its biochemistry, behavior and physiology, and many studies on its host plant *Nicotiana*, yet few studies have investigated *Manduca* host-plant interactions at the molecular level. Although *Manduca* larvae specialize on nightshade (Yamamoto and Fraenkel, 1960), they can be reared on artificial diet as well as on non-solanaceous plants, such as *Brassica* spp. (Brassicaceae) under laboratory conditions (Boer and Hanson, 1984). Although such behavior would classify *Manduca* as an oligophagous species rather than a specialist species, the broad host plant range accepted in the lab is not well documented in the field.

In this study, we focused on three typical host plants of *Manduca*: *Nicotiana attenuata* (coyote tobacco), *Solanum lycopersicum* (tomato) and *Datura wrightii* (sacred datura), all belonging to the Solanaceae (nightshade) family, as well as the non-host plant *Brassica napus* (rapeseed). While the solanaceous host plants differ in their secondary metabolites and proteinaceous effectors, *B. napus* also uses a different class of metabolites, glucosinolates, as its major chemical defense (Fahey et al., 2001).

Plants of the nightshade family employ alkaloids, phenylpropanoids, flavonoids, and protease inhibitors to deter herbivores: *Nicotiana*, for example, produces the alkaloid nicotine, as well as trypsin inhibitors. Both of these have been shown to be effective defenses especially against generalist herbivores but can also impact specialist performance (Steppuhn and Baldwin, 2007). *Nicotiana* plants as well as artificial diet containing high nicotine concentrations inhibit the growth of both *Manduca* and the

Abbreviations: AM, antennae and maxillae; G, gut with Malpighian tubules; SG, silk gland (labial gland); W, whole insect; P450, cytochrome P450 monooxygenase; GST, glutathione S-transferase; UGT, UDP-glycosyl transferase; ABC, ATP-binding cassette; IMD, immune deficiency; JAK, janus kinase; STAT, signal transducers and activators of transcription; JNK, c-Jun N-terminal protein kinases; SOCS, suppressor of cytokine signaling; AMP, antimicrobial peptide; WAP, whey acid proteins; AFP, antifungal proteins; MAPK, MAP kinase; OR, olfactory receptor; OBP, odorant binding protein; OSN, olfactory sensory neuron.

* Corresponding author.
E-mail addresses: ckoenig@ice.mpg.de (C. Koenig), abretschneider@ice.mpg.de (A. Bretschneider), heckel@ice.mpg.de (D.G. Heckel), grosse-wilde@ice.mpg.de (E. Grosse-Wilde), Hansson@ice.mpg.de (B.S. Hansson), hvogel@ice.mpg.de (H. Vogel).

[1] These authors contributed equally.

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polyphagous *Helicoverpa zea* (Harvey et al., 2007; Voelckel et al., 2001), and trypsin inhibitors from sweet potato have been shown to affect the growth of and therefore confer resistance to *Spodoptera litura* (Yeh et al., 1997). *S. lycopersicum* uses tomatine, chlorogenic acid, polyphenol oxidase, and proteinase inhibitors to deter herbivorous insects (Kennedy, 2003). Tomatine, a glycoalkaloid, has strong negative effects on the growth rate of *Spodoptera exigua* as well as of *H. zea*, both of which are generalist species that naturally occur on tomato (Bloem et al., 1989). *Datura wrightii* synthesizes the alkaloids scopolamine and hyoscyamine (Hare and Walling, 2006; Parr et al., 1990). Scopolamine prolongs the development and enhances the mortality of *Spodoptera frugiperda*, another generalist from the Noctuidae family (Alves et al., 2007).

Unlike the alkaloid-based chemical defenses of Solanaceae, brassicaceous plants use a system that is activated by two components, glucosinolates and myrosinase enzymes, as their major chemical defense (Bruce, 2014). Highly adapted insect herbivores can feed with impunity on their glucosinolate-containing host plants, but most polyphagous herbivores are negatively affected by high levels of glucosinolates (Arany et al., 2008; Kliebenstein et al., 2005; Winde and Wittstock, 2011).

Although transcriptional responses of generalist herbivores to different host plants or isolated toxins and of generalist and specialist herbivores to individual host plants have been analyzed, studies on large-scale transcriptional responses of herbivorous insects to a range of host plants are scarce or focused on aspects other than herbivore-host plant interactions (Zhan et al., 2011). Not only does host plant chemistry have an impact on detoxification-related gene expression, but secondary metabolites can be crucial for continued larval feeding. The chemical perception of the environment provides information on, for example, food sources and mating partners (Hanson and Dethier, 1973). Olfaction is important for larval plant discrimination and differences in host plant chemistry could potentially be reflected at the level of larval gustatory and olfactory gene expression. Likewise, plant secondary metabolites can influence an insect's immune system, resulting in the differential expression of immune-related genes. Immune defense strategies might also vary with the breadth of an organism's diet (Barthel et al., 2014; Lee et al., 2008; Ponton et al., 2013). Similarly, differences in both types and densities of host plant-associated bacteria can have an impact on innate immunity in herbivorous insects (Freitak et al., 2007).

The goal of this study was to compare the performance of larvae feeding on host and non-host plants as well as global changes in the gene expression of *Manduca* larval tissues elicited by feeding on these plants. To investigate these transcriptional responses, we used a replicated RNAseq approach combined with the official *Manduca* Gene Set (OGS2). In our analyses of the transcriptional responses of *Manduca* larvae, we mainly focused on putative detoxification-related, immune-related and olfactory genes. Here we show that *Manduca* larvae grow fastest on *Nicotiana*, one of the main host plants of this specialized insect. However, *Manduca* larvae performed equally well when fed on the non-host plant *Brassica* as when they fed on other host plants. We report specific changes in the expression of genes related to detoxification, immunity and olfaction as a consequence of feeding on different plants, providing insights into the plastic response of an herbivorous insect with a restricted repertoire of host plants.

2. Material and methods

2.1. Biological material and Manduca sexta rearing

Wild types of the following plant species were used for the experiments: *N. attenuata*, *Datura wrightii*, *S. lycopersicum* (cv. Balcony Magic) and *B. napus* (cv. Dwarf Essex). All plants were grown in a greenhouse maintained at 26 °C, 75% humidity, and a 16 h light and 8 h dark cycle.

Manduca larvae were fed on artificial diet (46 g of agar, 144 g of wheat germ, 140 g of corn meal, 76 g of soy flour, 75 g of casein, 24 g of Wesson's salt mixture, 36 g of sugar, 5 g of cholesterol, 12 g of ascorbic acid, 6 g of sorbic acid, 3 g of methyl paraben, 9 mL of linseed oil, 60 mL of 3.7% formalin, 30 mg of nicotinic acid, 15 mg of riboflavin, 7 mg of thiamine, 7 mg of pyridoxine, 7 mg of folic acid, and 0.6 mg of biotin per 1.8 L of water). Insects were kept at 26 °C, 75% humidity, and a 16 h light and 8 h dark cycle.

2.2. Feeding assay

For the feeding assay, larvae were reared on artificial diet up until shortly before reaching the third instar (L3), when they were transferred to one of the four host (*Nicotiana*, *Datura*, *Solanum*) or non-host (*Brassica*) plants, respectively, or fed the artificial diet. Plants were 5–10 weeks old (flowers had been removed) and larvae were allowed to feed on whole plants or the artificial diet for eight consecutive days (Fig. 1A). Larval weight was recorded every second day. Differences in the development were statistically analyzed using ANOVA (in R). From the average weight per treatment, we calculated the relative growth rate, since it represents the proportional increase in mass per unit time and adjusts for initial size and the nonlinear patterns of growth over time.

Silk (labial) glands (SG), as well as guts together with Malpighian tubules (G), were dissected from L4 non-molting larvae at Zeitgeber time 8–12. Antennae together with maxillae (AM) were collected from L5 non-molting larvae at Zeitgeber time 8–12. Insects were dissected in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). In addition, whole insects (L4 non-molting larvae) (W) were ground in liquid nitrogen at Zeitgeber time 8–12 for RNA isolation. Dissected tissue was kept at −20 °C in lysis buffer (innuPREP RNA Mini Kit, Analytik Jena, Germany) until being used for RNA isolation.

2.3. RNA isolation and illumina sequencing

RNAseq experiments were carried out with RNA isolated from larvae reared on artificial diet or different host plants. For SG, G, and W samples, three larvae were pooled for each RNA sample (biological replicate). For AM samples, ten larvae were pooled to receive a sufficient amount of RNA. Three replicates were created from each tissue. Total RNA was extracted according to the manufacturer's instructions (innuPREP RNA Mini Kit, Analytik Jena, Germany).

Library construction and sequencing was performed by the Max Planck Genome Center Cologne, Germany (http://mpgc.mpipz.mpg.de/home/). 1 μg of total RNA was used for a TruSeq RNA library and mRNA enrichment was performed. The library was sequenced with an Illumina HiSeq2500 sequencer. Approximately 10 million 100 bp single-end reads per biological replicate, per treatment, and for each of the tissue samples were obtained. Quality control measures, including filtering high-quality reads based on the score given in fastq files, removing reads containing primer/adaptor sequences and trimming read length, were carried out using CLC Genomics Workbench v6.5 (http://www.clcbio.com).

2.4. Gene annotation

The *Manduca* OGS2-predicted gene set was annotated using BLAST, Gene Ontology and InterProScan searches using BLAST2GO PRO v2.6.1 (www.blast2go.de) (Conesa and Götz, 2008). For BLASTX searches against the non-redundant NCBI protein database (NR database) up to 20 best NR hits per transcript were retained, with

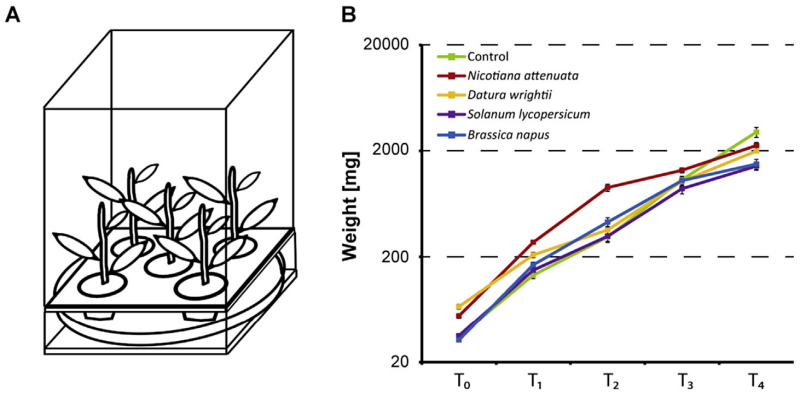


Fig. 1. A) Experimental setup of feeding assay. Larvae were reared on five non-flowering plants (both host and non-host plants) for eight consecutive days. B) *M. sexta* larval development on host and non-host plants. P-value: * < 0.05, ** < 0.01, *** < 0.001 (ANOVA); N = 20/plant or diet. T: timepoint.

an E-value cut-off $\leq 10^{-1}$ and a minimum match length of 15 amino acids to obtain the best homologue also for predicted short polypeptides. Annex (Myhre et al., 2006) was used to optimize the GO term identification further by crossing the three GO categories (biological process, molecular function and cellular component) to search for name similarities, GO term relationships and enzyme relationships within metabolic pathways (Kyoto Encyclopedia of Genes and Genomes). The *Manduca* OGS2 data can be accessed from https://i5k.nal.usda.gov/Manduca_sexta or ftp://ftp.bioinformatics.ksu.edu/pub/Manduca/OGS2/.

2.5. Digital gene expression analysis

Digital gene expression analysis was carried out by using QSeq Software (DNAStar Inc.) to remap the Illumina reads from all samples (each of the replicates of all samples was mapped individually) onto the reference backbone (*Manduca* Official GeneSet2) and then by counting the sequences to estimate expression levels, using previously described parameters for read mapping and normalization (Vogel et al., 2014a). Biases in the sequence datasets and different transcript sizes were corrected using the RPKM algorithm (reads per kilobase of transcript per million mapped reads) to obtain correct estimates for relative expression levels. To control for the effect of global normalization using the RPKM method, we also analyzed a number of highly-conserved housekeeping genes that are used as control genes for quantitative PCR. These included several genes encoding ribosomal proteins (rpl3, rpl4, rpl13, rpl15, rps2, rps8, rps12, rps15a, rps18 and rps24), elongation factor 1alpha and eukaryotic translation initiation factors 4 and 5. The corresponding genes were inspected for overall expression levels across samples and treatments, and were found to display expression level differences (based on RPKM values) lower than 1.3-fold between samples, indicating they were not differentially expressed and validating their housekeeping function. In Supplementary data Table 1 (Table S1) all of the gene names mentioned in the tables and figures of the main manuscript as well as Supplementary Data are linked to the respective Msex2 official gene identifiers.

Table 1
Growth rate of *Manduca sexta* larvae fed on host- and non-host plants, as well as on artificial control diet. T: timepoint.

Treatment	T_1	T_2	T_3	T_4
Control	0.28	0.18	0.27	0.22
Nicotiana attenuata	0.35	0.26	0.08	0.12
Datura wrightii	0.24	0.13	0.23	0.13
Solanum lycopersicum	0.30	0.16	0.22	0.10
Brassica napus	0.36	0.20	0.20	0.08

3. Results

3.1. Growth rate on different plants

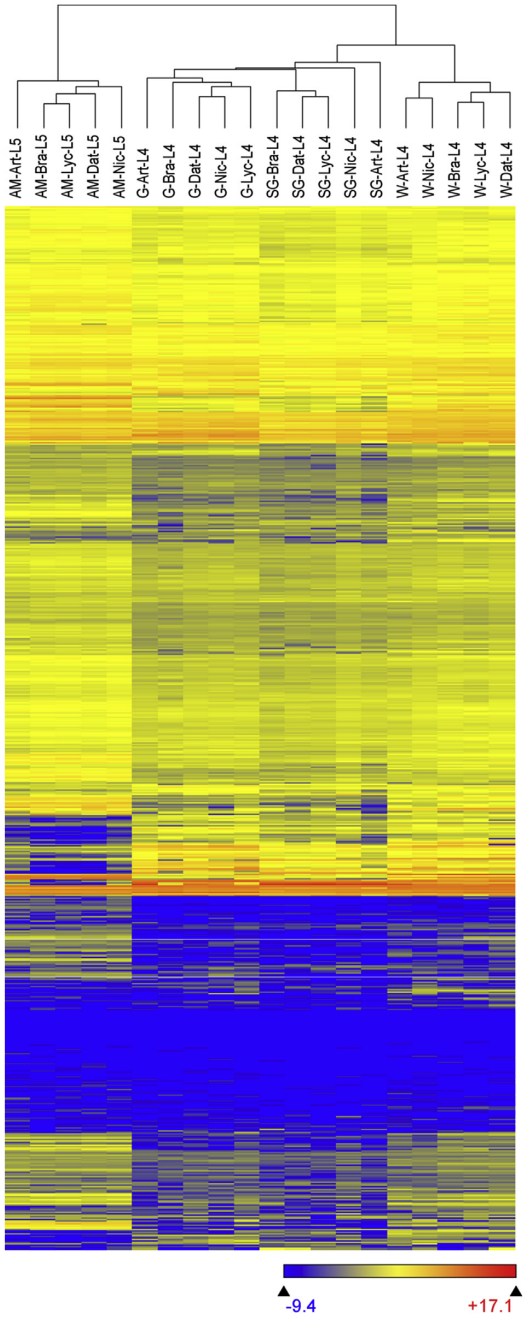
All larvae (L3) were weighed at day 0 and every second day following. Control animals were fed artificial diet and exhibited consistent growth and development, showing that this is a suitable control.

The larvae developed differently on the respective plants, both at the beginning and during the course of the experiment. The start weight of animals fed on *Datura* and *Nicotiana* was significantly different from the start weight of animals fed on the other plants (time point 0; p-value < 0.001; Fig. 1B; S1). Until the fourth day, larvae fed on *Nicotiana* were significantly heavier than those fed on all other plants (time point 2; p-value < 0.001). However, two days later (time point 3) there was no significant difference between the larvae fed on the plants and those fed on the control diet. At the last time point sampled, larvae performed equally well when fed on the control diet and on *Nicotiana* (p-value > 0.05) and better than those fed on all the other plants (p-value < 0.05). Even though larvae fed on *Datura* were significantly heavier at the beginning of our measurements, they were unable to retain their body mass to the degree that larvae fed on the other plants were. After four days, larvae fed on *Datura* were as heavy as those fed on tomato or rapeseed. Both the tomato host plant and the non-host rapeseed, however, led to a similar development (measured as growth rate) over time of *Manduca* larvae. Interestingly, larvae fed on *Brassica* show the highest growth rate at time point 1, which subsequently significantly decreased (Table 1). The control individuals fed on artificial diet developed consistently throughout the measured time points.

In summary, *Manduca* larvae have a faster initial growth rate when fed on *Nicotiana* and attain a higher final weight when fed on artificial diet. However, *Manduca* performs nearly as well when fed on *Brassica*, an acceptable non-host plant compared to the host plants *Datura* and *Solanum*.

3.2. Genome expression profile and GO enrichment analysis

We mapped the sequencing reads obtained from the individual samples (tissues and whole larvae fed on different host plants) to the official gene set of the *Manduca* genome project containing



. **2.** Heatmap depicting all identified and mapped *M. sexta* OGS2 genes across tis-s and treatments. The different tissues cluster each together in individual clades. 2-transformed RPKM values are shown (blue resembles low expressed genes, while represents high expressed genes).

15,540 predicted genes (excluding splice variants). The sequence for cytochrome P450 monooxygenase CYP6B46 was added manually, as it was not present in the official gene set but is known to be regulated when *Manduca* feeds on *Nicotiana* (Kumar et al., 2014). Fig. 2 shows a heat map of the log2-transformed expression values (RPKM) of all genes for all investigated tissues. We performed hierarchical clustering using Euclidean distance metric and the centroid linkage method. As expected, each tissue forms an individual cluster based on its gene expression profile. Samples from larvae fed on artificial diet form a distant branch separate from larvae fed on plants with regard to the antennae/maxillae tissue (AM), silk (labial) gland tissue (SG), and gut/Malpighian tubules tissue (G). As a notable exception to the above patterns, in the samples of whole insects (W), larvae reared on *Nicotiana* and artificial diet cluster together, suggesting that feeding on these two diets seems to influence global gene expression patterns in the same way.

Using as a conservative cut-off a minimum 8-fold change between samples, we compared the frequencies of gene ontology (GO) terms of larvae fed on plants to the frequencies of those reared on artificial diet. Larvae grown on each of the plants exhibited an increase in GO terms linked to vesicle-mediated transport, structural composition of cuticle, and transcription factors. In addition, in larvae reared on *Brassica*, GO terms such as fatty acid synthase complex, tetrapyrrole binding, cell differentiation, enzyme regulator activity, and actin filament based process were overrepresented (Fig. S2A). In larvae fed on *Datura*, GO terms such as tetrapyrrole binding, neurotransmitter binding, monooxygenases, oxidoreductases, cell differentiation, glutathione metabolic process, and enzyme regulator activity were overrepresented (Fig. S2B). In larvae fed on *Solanum*, GO terms such as tetrapyrrole binding, actin-filament-based process, and locomotion are overrepresented (Fig. S3A), and in larvae fed on *Nicotiana*, GO terms such as transferase activity and locomotion were overrepresented in addition to the ones mentioned earlier (Fig. S3B).

The total number of differentially expressed genes across all tissues is the greatest in the G samples, especially genes that are related to detoxification (Table 2). However, except for one tissue and category (olfaction in SG), genes of the three functional categories we focused on are regulated in response to plant feeding in a tissue and treatment-specific manner.

Next, we compared gene expression in the W samples from individuals fed on the four plants to gene expression in individuals fed on artificial diet. We found 322 genes at least 4-fold up-regulated (p-value < 0.05) in the samples of larvae fed on plants (Table 3).

3.3. Detoxification-related genes

The detoxification and metabolism of most xenobiotics likely occurs via a common set of detoxification-related enzymes, all of which belong to multigene families. Phase I enzymes, including cytochrome P450 proteins (P450s), participate in the functionalization step of xenobiotic detoxification, whereas Phase II enzymes, such as glutathione S-transferases (GST) and UDP-glycosyltransferases (UGTs), convert lipophilic xenobiotics into more hydrophilic compounds to facilitate excretion, for example by ATP binding cassette (ABC) transporters, or sequestration during phase III.

3.3.1. Glutathione S-Transferases

Glutathione S-transferases (GST), which are part of Phase II detoxification, catalyze the conjugation of glutathione to xenobiotics (Eaton and Bammler, 1999). The total number of predicted GSTs in the *Manduca sexta* genome was 42 and included several

Table 2
Total number of differentially expressed genes in all tested tissues (P value < 0.05). AM: antennae and maxillae; G: gut with Malpighian tubules; SG: silk gland (labial gland); W: whole insect.

Tissue	Total# of DEGs	Related function		
		Detoxification	Immunity	Olfaction
AM	269	4	2	6
Gut	1711	47	16	1
SG	325	2	3	0
Whole larvae	758	44	5	6

subfamilies, such as the delta, epsilon, omega, sigma, theta, zeta and microsomal (MAPEG (Membrane Associated Proteins in Eicosanoid and Glutathione metabolism) domain containing) GSTs. We were able to identify 40 transcripts in our data set.

Overall, samples show that GST gene expression patterns cluster in a tissue-specific manner, except for in one case (W-Dat-**L4**, Fig. S4). Thus, GST expression patterns are quite homogeneous across the samples, with notable exceptions. For example, GST delta**4** is up-regulated in the G tissue of *Manduca* larvae fed on *Datura* and in the SG samples of *Manduca* larvae fed on *Nicotiana* (Fig. S4). This gene could be important for successful larval development on *Datura* or *Nicotiana*, and it could either be involved in xenobiotic detoxification or be a more general cellular stress response.

In general, members of the epsilon subfamily are related to the detoxification of xenobiotics (Corona and Robinson, 2006). *Manduca* GSTs Epsilon2 and 3 are similarly up-regulated across all G samples derived from plant-fed larvae, unlike in the G samples from larvae fed on artificial diet. In larvae fed with artificial diet, none of the GSTs were up-regulated compared to samples from plant-fed larvae. This expression pattern implies that genes of this family and especially the two above-mentioned epsilon subfamily members do not have a clear plant-specific function.

The microsomal GST MAPEG1 is most expressed in AM tissue, whereas MAPEG2 is equally expressed in all samples. Since those two genes do not show a treatment-specific up-regulation, they might not be involved in host-plant detoxification; instead, they might play a role in more general cellular processes.

The GST Sigma1 is thought to be involved in oxidative stress response in *Drosophila melanogaster* (Singh et al., 2001). Our data show that, although the *Manduca* orthologue is generally up-regulated in the AM and G tissue of larvae fed on plants, this up-regulation is more pronounced in the *Brassica*-fed larvae. Feeding on *Brassica*, the only non-host plant used in this assay, might lead to higher oxidative stress levels in *Manduca* larvae. The expression profile of GST Sigma 5 is similar to that of GST Sigma 1. It remains to be elucidated whether those two genes are specifically involved in the ability to develop on *Brassica* or whether they are involved in a more general stress response and are induced as a result of the breakdown of glucosinolates into their toxic end products.

3.3.2. ABC transporters

ATP-binding cassette (ABC) transporters constitute one of the most abundant protein families in all organisms (Sturm et al., 2009). Those transmembrane proteins can be divided into different subfamilies (A-H) with different functions. They are involved in cellular transport mechanisms and Phase III detoxification, as well as in general cellular processes (Sturm et al., 2009).

We identified 54 distinct contigs in the *Manduca* transcriptome that are part of ABC transporters (53 of which were annotated in OGS2). The annotated gene model for ABC-D1 and ABC-D2 was split in OGS2; therefore the N- and C-terminal halves were mapped individually. Many members of the subfamilies identified showed a treatment- or tissue-specific expression profile (Fig. 3). In contrast to the distinct tissue-specific clustering observed for the GST genes, clustering of the ABC transporter genes is less clear. For example, SG samples from larvae fed on plants do not form a separate cluster, but rather display treatment-specific gene expression patterns. In contrast, AM samples form a distinct cluster separately from all other samples. While most ABC genes display a rather homogeneous expression pattern within a specific tissue and across treatments, there are some notable exceptions.

Members of subfamilies B and C are involved in detoxification processes and multidrug resistance (Liu et al., 2011). Transcripts homologous to ABC-B1 and ABC-B3 are significantly more highly expressed (p-value $_{B1} = 0.016$; p-value $_{B3} = 0.028$) in the G of insects fed on plants than in the G of larvae fed on artificial diet. ABC-B1, also known as PGP or MDR1, is known for conferring resistance to many different xenobiotics (Holland et al., 2003). Since it is up-regulated most in insects fed on *Datura* and *Solanum*, this plant-specific expression pattern might be an adaptation of *Manduca* larvae to those plants. ABC-B3 is important for antigen processing in mammals (Holland et al., 2003). However, although its function in insects is still unknown, it is highly expressed in the G when larvae are fed on plant material (Fig. 3) and might thus have an important role when insects are exposed to plant secondary metabolites or to general and abundant plant compounds. The transcripts of ABC-C3 and ABC-C11 are most highly expressed in the AM tissue but do not display treatment-specific expression patterns (p-value = 0.665). ABC-C3 is known for its ability to remove toxic organic ions (Zelcer et al., 2001), and its high expression in AM samples compared to other tissues suggests that either the detoxification of xenobiotics or more general transport processes starts within the larval mouth and that this response might be general and dietary-independent. ABC-C2 and ABC-C6 were more expressed in specific tissues and in a treatment-specific way, suggesting these genes could be involved in the development of larvae fed on *Brassica Datura* or on *Solanum* respectively (Fig. 1B). In addition to the above ABC-C transporters, ABC-C4 (Fig. 3), a transporter which has been shown to confer resistance to several xenobiotics (Russel et al., 2008), displays a peak in gene expression (7652 up-regulated) in the W and G samples from *Manduca* larvae fed on *Solanum*, and may be an important developmental adaption to this specific host plant.

Proteins belonging to the ABC-D subfamily transport long-chained-fatty acids (Theodoulou et al., 2006). The respective *Manduca* ABC-D genes are mainly expressed in the mouth parts as well as in the G tissue (Fig. 3) and display overlapping expression profiles with the ABC-A genes, which are involved in cholesterol transport. Those two subfamilies interact in the fatty-acid digestion by transporting these fatty acids to the gut cells; there they are reassembled into triglycerides and coated with cholesterol before being transported into the blood stream (Dean and Annilo, 2005).

Table 3
Differential gene expression from whole (W) larvae reared on plants or on artificial control diet.

Comparison	W-Bra vs W-Art	W-Dat vs W-Art	W-Lyc vs W-Art	W-Nic vs W-Art	total
DEG 4 fold, p < 0.05	174	256	170	49	322
Treatment specific	9	83	29	11	132

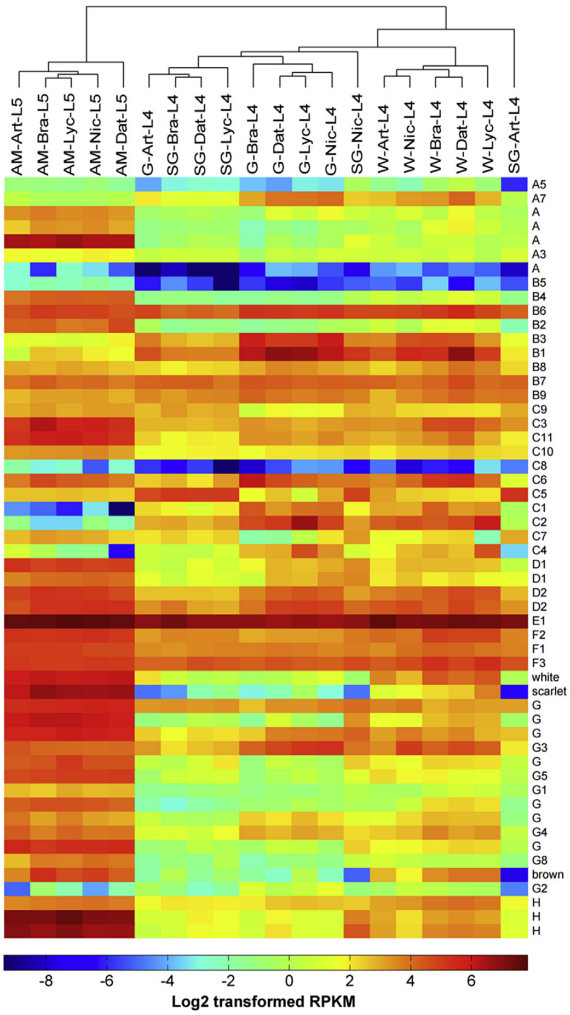


Fig. 3. Gene expression of ABC transporters when *Manduca sexta* larvae were shifted from control diet to plants. Values are based on log2-transformed RPKM values relative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). AM: antennae and maxillae; G: gut with Malpighian tubules; SG: silk gland (labial gland); W: whole insect.

Members of subfamily G, which has many functions besides detoxification (Holland et al., 2003), were found to be mostly expressed in the mouth parts. The major eye pigment precursor transporters *white*, *brown* and *scarlet* (Chen et al., 1996), as well as ABC-G5 and ABCG8, both of which are involved in sterol transport (Lee et al., 2001), are mainly expressed in the AM samples. Although the function of the transporter ABC-G3 is not yet understood, it is mainly expressed in the G tissue of insects fed on plants and therefore might exhibit a general function for developing on plants. The function of ABC transporters belonging to subfamily H is not yet known. In *Manduca*, ABC-H transporters are mainly expressed in the AM tissue, which suggests a general function rather than specific involvement, for example in detoxification (Fig. 3). Overall, ABC transporters might be an important part of larval *Manduca* plastic response and adaptation to the different host plants.

3.3.3. *UDP-glycosyltransferases*

UDP-glycosyltransferases (UGTs) transfer sugars to endogenous and exogenous compounds. They catalyze the biotransformation of xenobiotics, which supports insects in detoxifying dietary metabolites (Ahmad and Hopkins, 1993). Additionally, the transfer of sugars play a role in processes such as pigmentation, cuticle formation and olfaction (Wang et al., 1999).

The OGS2 of *Manduca* comprises 42 annotated UGTs. We identified three additional candidate genes in the transcriptome but none of these were expressed in the investigated tissues. UGTs are expressed in all investigated tissues (Fig. S5). The expression pattern of UGTs is similar in G and SG samples. The expression of three UGTs (39B3, 40H2, 40L2) was focused on the AM samples. The complete UGT gene expression data showed that the tissues generally cluster together, independent of the treatment. However, the G and SG samples from larvae fed on artificial diet are distant from samples of larvae fed on plants. Two UGTs (40C2, 340A3) are up-regulated in the G tissue depending on the treatment (p-value < 0.05) and might thus play a role in the detoxification of plant-specific xenobiotics. In addition to seven other UGTs (p-value < 0.05; 33C, 40C1, 40E2, 40J1p, 42A3, 42A4, 50A3), UGTs 40C2 and 340A3 also exhibit a differential expression pattern in the W samples. Although generally expressed at low levels, one UGT (33H1) is differentially expressed in the AM tissue (p-value < 0.05). We did not detect statistically significant differences in the expression of UGTs in the SG tissue between larvae fed on plants and larvae fed on artificial diet.

3.3.4. *Cytochrome P450 monooxygenases*

Cytochromes P450, which are membrane-bound enzymes involved in the metabolism of a variety of molecules such as vitamins and hormones, are probably best known for their ability to metabolize xenobiotics and have frequently been shown to be inducible when insects are exposed to certain plant metabolites (De la Paz Celorio-Mancera et al., 2011; Feyereisen, 1999; Hung, 1997; Yamamoto et al., 2010). The official gene set of the *Manduca* genome contains 99 annotated P450 sequences. We added the sequence of CYP6B46 because it has recently been reported as being induced when *Manduca* larvae are fed on *Nicotiana* wild-type plants which contain the ability to produce nicotine (Kumar et al., 2014). While performing a BLAST search with the transcriptome, we identified 17 additional candidates; Figure S6 thus shows the expression profiles of 117 putative P450 genes.

Although several P450s were not expressed in any of the tissues and samples analyzed, a number of P450s were specifically expressed in the AM, SG, or G tissues (not, however, in a clan-specific manner). Several P450s displayed high expression levels only in the W samples, suggesting that they are expressed in tissues other than those investigated (Fig. S6). All AM as well as W samples showed similar expression profiles and cluster together. The G as well as the SG tissue from larvae fed on *Brassica*, *Datura*, and *Solanum* cluster together, but the samples from larvae fed on *Nicotiana* and artificial diet are distinct. These P450 gene expression patterns match the patterns observed for the larval development on the respective plants (Fig. 1B).

19 and 22 P450s were differentially expressed in the G and in W samples, respectively, depending on which plants larvae fed on (p-value < 0.05). Although four of these P450s were not characterized further, three belong to the CYP4 clan; this clan plays an important role in biosynthesizing hormones and pheromones as well as in metabolizing plant toxins such as isoquinoline alkaloids (Danielson et al., 1998). Seven P450s belong to the CYP3 clan, members of

which have been shown to metabolize a broad spectrum of plant compounds such as sesquiterpenes, flavonoids, and insecticides (Feyereisen, 2006; Hung, 1997). One of these CYP3 P450s is also differentially expressed in the SG samples (p-value = 0.0475). Four P450s belong to the clan of mitochondrial P450s, which are typically up-regulated when larvae are exposed to xenobiotics (Yamamoto et al., 2010); a differentially expressed P450 belongs to the CYP2 clan involved in ecdysteroid metabolism in insects.

In larvae fed on *Brassica*, cytochrome P450 CYP332A4 was more than 4-fold up-regulated. Although this gene has already been identified in the *Manduca* midgut transcriptome (Pauchet et al., 2010), its function in relation to the detoxification of host plant metabolites is unknown.

CYP6B46 was previously reported to be induced in the guts of *Manduca* larvae feeding on *Nicotiana* (Kumar et al., 2014). Although we could confirm the up-regulation of CYP6B46 in larvae fed on nicotine (p-value = 0.0477), this P450 gene is expressed even more highly in larvae fed on all other tested plants compared to larvae fed on both *Nicotiana* and artificial diet. Unlike most of the other genes identified in the tissues of larvae fed on all types of plants, expression of P450s seem not to be affected in the AM tissue of the larvae that fed on any of the plants.

3.4. Immunity

The microbiota and potential pathogens which are encountered by *Manduca* larvae when feeding on different plant species can be quite variable and have to be dealt with by their immune system. The multilayered innate immune system of insects is made up of pattern recognition (pattern recognition receptors (PRRs)), signal transduction pathways, and antimicrobial peptides (AMPs) (Casanova-Torres and Goodrich-Blair, 2013) and other defense-related proteins (Hoffmann, 1995; Ratcliffe et al., 1985: Kanost et al., 2004) whose release can be effective against pathogenic challenges (Boutros et al., 2002; Kanost et al., 2004; Ma and Kanost, 2000). In insects, these processes are regulated by the Toll, JAK/STAT, IMD and JNK pathways (Boutros et al., 2002; Dostert et al., 2005; Lemaitre et al., 1996, 1995; Zhong et al., 2012). These pathways help insects defend themselves against invading microorganisms; the Toll pathway is stimulated by gram-positive bacteria as well as by fungi, whereas the IMD pathway is mainly stimulated by gram-negative bacteria (Lemaitre et al., 1997). The hierarchical clustering of the immune-related gene data resulted in 3 main blocks containing AMs, G and SGs, as well as W samples (Fig. 4).

In addition to genes from the Toll and IMD pathway, we could identify larger parts of the JAK/STAT pathway, such as domeless, hopscotch, STAT and SOCS. Genes from those pathways were found to be expressed evenly and at moderate levels in all larval tissues and regardless of what plants larvae fed on (Fig. 4). However, for a number of those signaling pathway genes, we observed high expression levels both in W as well as in the AM samples.

Unlike the even and moderate expression levels of the pattern recognition and signaling pathway genes in larvae, most of the antimicrobial peptides (AMPs) are expressed at low levels in most of the tissues, display a more dynamic regulation and are differentially expressed in a diet-dependent way. Our quantitative analysis of transcripts showed that the majority of the AMPs were detectable in the W samples, which includes the integument and the fat body, and is the major organ for AMP synthesis during systemic immune responses (Ferrandon et al., 2007). In general, all the tissues of larvae fed artificial diet treatments displayed the overall lowest expression of AMPs.

Several AFPs - antifungal proteins - were found to be most highly expressed in the larval mouthparts when larvae fed on plants (Fig. 4), but these increases were not specific to any one plant. However, in other tissues, such as the SG as well as in W samples, a number of AFPs, attacins, a cecropin and gallerimycin displayed statistically significant differences between treatments; in other words, host plant-specific expression patterns were observed. Compared to other AMPs, the whey acid proteins (WAPs) are highly expressed in the G and SG, and display expression differences related to dietary differences of the larvae in a complex tissue and host-plant specific way.

Members of the MAP kinase (MAPK) family are involved in stress response and the regulation of immune responses (Botella et al., 2001). A total of 16 MAPK pathway genes are present in the *Manduca* OGS2, eight of which are expressed in our transcriptome data (Fig. 4). Two of those MAPK genes were differentially expressed in the G samples (p-value < 0.05).

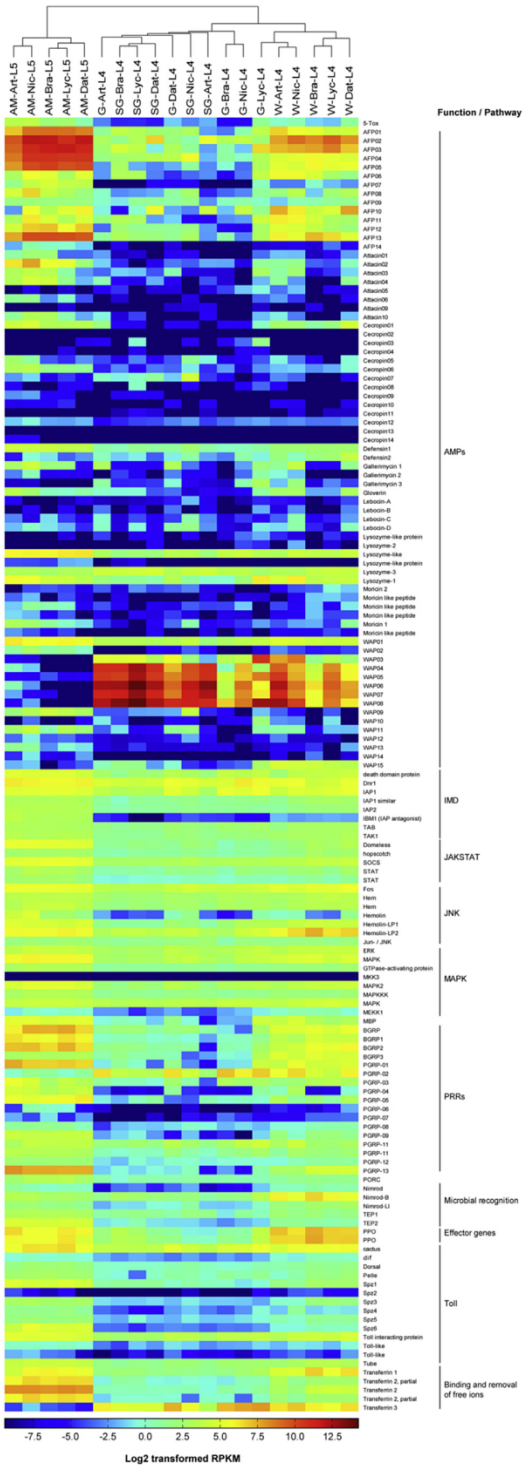
An important aspect of the immune system is melanization and wound healing. Phenoloxidases (POs) are involved in the biosynthesis of melanin and are therefore considered to be essential components of the insect immune system (Sugumaran, 2002). The *Manduca sexta* genome includes two phenoloxidases, both of which are mainly expressed in the W and AM samples and are not significantly differentially expressed between treatments (p-value$_1$ = 0.827; p-value$_2$ = 0.952).

3.5. Olfaction

Two major gene families involved in insect olfaction code for olfactory receptors (ORs) and odorant-binding proteins (OBPs) (Hansson and Stensmyr, 2011). ORs are expressed by olfactory sensory neurons (OSNs) in the antennae, where the OR proteins are situated in the dendritic membrane housed by the olfactory sensilla (Vosshall et al., 2000). ORs detect volatile ligands, mediating a neuronal response. In all cases to date, ORs are co-expressed with the olfactory receptor coreceptor ORCo (Vosshall and Hansson, 2011); ORs and ORCo form a functional, heteromultimeric complex (Sato et al., 2008; Wicher et al., 2008). OBPs are globular, water-soluble proteins that are secreted into the sensillum lymph surrounding the OSN dendrite. They supposedly bind to lipophilic odorant molecules, enabling them to transition into the sensillum lymph and therefore to the OR. OBPs have also been reported to be involved in non-olfactory functions in other body parts (Pelosi et al., 2006).

3.5.1. Odorant receptors

We annotated 70 OR coding genes as well as the ORCo gene within the genome data of *Manduca* (Koenig et al., forthcoming). The sequences of MsexOR-10 and MsexOR-15 were added to the *Manduca* gene set, because although they are annotated in the genome, they were missing in the final OGS2. Using the RNAseq dataset presented here, we analyzed the expression of the respective gene families in larval tissues, as well as differences in gene expression between larvae fed on different diets. In total, 20 OR genes and ORCo are expressed in the AM tissue of larvae (Fig. 5; RPKM ≥0.25: MsexOR-11, 18, 23, 24, 26, 27, 29, 30, 32, 35, 47, 51, 52, 57, 67, 71, 72, 74, 75, 76). Especially noteworthy among the expressed ORs was MsexOR-51, which in adult antennae is male-specific and belongs to the Lepidoptera pheromone clade (Koenig et al., forthcoming). Although no response of *Manduca* larvae to female emitted pheromones has yet been reported, such a response has been described for *Spodoptera littoralis* (Poivet et al., 2012). The other two reported pheromone receptor candidates, MsexOR-1 and MsexOR-4 (Grosse-Wilde et al., 2010; Patch et al., 2009), are not expressed in the larvae. Regarding other expressed receptors, we can only speculate on function. MsexOR-30, for example, is an orthologue of BmOR-56, the OR in *Bombyx mori* that responds to cis-jasmone and mediates the attraction of the larva to mulberry



4. Expression of immunity-related genes. Gene expression levels in the different ues are based on log2-transformed RPKM values, and responses are expressed ative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). AM: antennae and maxillae; G: guts with Malpighian tubules; SG: silk gland (labial gland); W: whole insect.

leaves (Tanaka et al., 2009). Other *Manduca* ORs are also orthologues of larval *B. mori* ORs (see Table 4 for receptors and detected compounds). For four ORs (MsexOR-42, 47, 57, 66), expression could also be detected (RPKM $\geq$ 0.25) in non-olfactory larval tissue. RPKM values for all OR genes are low, indicating low transcript abundance within the tissue when compared with all other gene families analyzed. Notably, the antennae of *Manduca* larvae contain presumably seven olfactory sensilla (six basiconic and one styloconic), and the maxilla contains thirteen presumably olfactory sensilla (nine basiconic on the palp, two basiconic and two stylocinic on the galea) (De Boer et al., 1977; Dethier and Schoonhoven, 1966; Kent and Hildebrand, 1987). In total, 30–35 OSNs are present in the tissues, which fits the number of ORs expressed according to our data. The number is comparable to the number of ORs expressed in larval chemosensory tissues of *S. littoralis* (20 ORs) (Poivet et al., 2013) and *B. mori* (23 ORs) (Tanaka et al., 2009). Although the RPKM values fluctuated between the samples from larvae on different host plants, the differences are only statistically significant for MsexOR-27 (p-value = 0.032).

3.5.2. Odorant-binding proteins

There were 49 annotated OBP coding genes in the genome. Expression of eleven OBPs was restricted to the AM tissue in larvae (ABP2, 4, 6, OBP5, 11, 12, 13, 14, 15, 31, 37). Twelve OBPs were expressed in all investigated tissues (ABP1, 7, 8, x, OBP21, 22, 25, 26, 30, 35, 38, 40). For ten OBPs, we did not detect expression in any of the analyzed tissues (ABP3, OBP1, 2, 3, 4, 16, 23, 24, 27, 29). Unlike most ORs, several OBPs were present in G, as well as SG tissue (Fig. 6).

Five OBPs (ABP6, OBP17, OBP28, OBP32, and OBP38) were differentially expressed in the AM depending on larval diet (p-value < 0.05). OBP33 was up-regulated in G tissue when larvae fed on *Solanum* (p-value < 0.05). Six OBPs (ABP7, OBP14, 17, 30, 32, 38) exhibited differential expression in W samples (p-value < 0.05). We did not detect significant expression changes for any OBPs present in the SG samples.

4. Discussion

The aim of this study was to compare *Manduca* larval developmental as well as transcriptional responses to host- and non-host plants. To address this, a developmental assay was combined with a replicated RNAseq approach, using *Manduca* larvae fed on different diets. Our results confirm that *Manduca* is capable of developing on different host and non-host plants. Furthermore, we demonstrate that overall transcriptional signatures of larvae fed on different host plants are quite specific for each host plant and larval tissue combination, suggesting larvae are capable of a plastic and adaptive response to each plant diet.

To feed successfully on host plants, larvae actually require several adaptations; these include host-plant perception and continued feeding (non-deterrency) through olfactory and/or gustatory cues, digestion and plant nutrient uptake processes, detoxification of plant secondary metabolites and countermeasures against other plant defenses, and the management of plant-associated microbiota. In addition to the tissues of whole larvae, we focused our transcriptomic analyses on a selected set of larval tissues, namely antennae and maxillae, which are the first tissues interfacing with plant material, followed by salivary glands and guts.

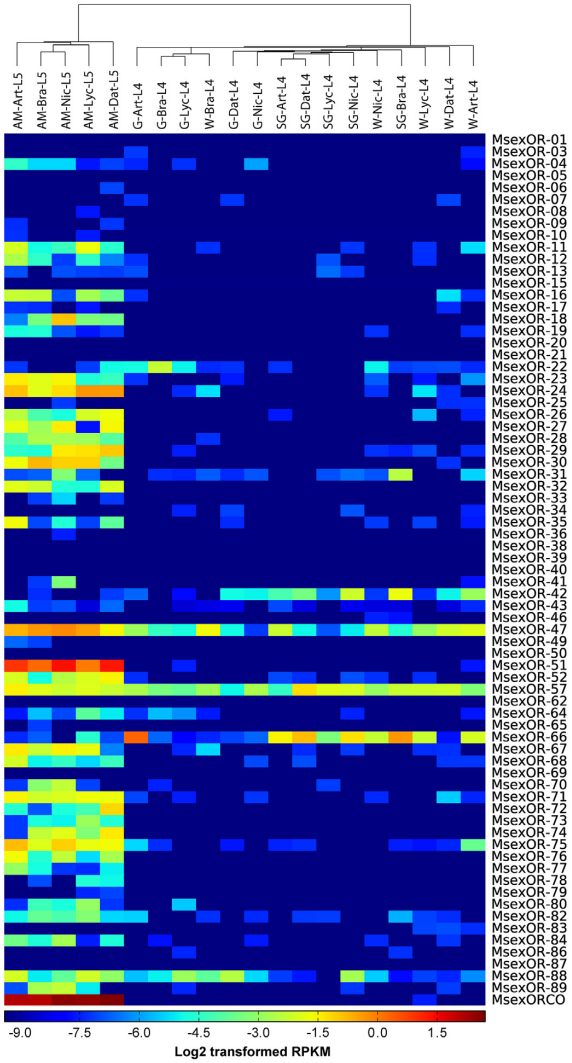


Fig. 5. Expression patterns of olfactory receptors (ORs). Values are based on log2-transformed RPKM values relative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). AM: antennae and maxillae; G: guts with Malpighian tubules; SG: silk gland (labial gland); W: whole insect.

Under laboratory conditions, *Manduca* can be reared on artificial diet and on plants belonging to several different plant families (Boer and Hanson, 1984). Our larval growth and performance assay suggests that *N. attenuata* is the best host plant for *Manduca*, better than *Datura wrightii*, *S. lycopersicum*, and the non-host plant *B. napus*. In our set of tested plants, the larvae grew fastest on *Nicotiana*. Artificial diet is rich in carbohydrates and low in toxic substances compared to a diet of plants and should therefore be considered the optimal "diet"; yet initial growth rates of larvae fed on *Nicotiana* were even higher than the values of those fed on artificial diet. A possible explanation could be that *Nicotiana* foliage contains optimally balanced nutrients required for larval development compared to the other tested plants or the artificial diet. Alternatively, growth differences could be due to the effect of hormesis, whereby a low amount of defensive compounds can have positive effects on the growth rate of insects (Harvey et al., 2007; Kaiser, 2003; Voelckel et al., 2001). Such a hormetic effect has been observed at the developmental as well as transcriptional level in *Helicoverpa armigera*, when larvae are fed low gossypol concentrations (De la Paz Celorio-Mancera et al., 2011). Plants of the *Nicotiana* genus are known to produce large amounts of the secondary metabolite nicotine as well as trypsin inhibitors, both of which inhibit the growth of many insect species (Baldwin, 2001; Steppuhn and Baldwin, 2007). However, the specialized insect *Manduca* seems to have overcome this barrier; not only do P450s probably form the basis for the metabolism of nicotine (Kumar et al., 2014), but ABC transporter-like mechanisms excrete nicotine into the hemolymph (Gaertner et al., 1998; Wang et al., 2005). In addition to data for *Manduca*, *high expression of a UGT was significantly linked to nicotine resistance in D. melanogaster* (Marriage et al., 2014). However, despite a likely role of secondary metabolites in *Manduca* larval performance, we cannot exclude the possibility that *Nicotiana* contains elevated levels of substances such as nitrogen-containing compounds, which have been shown to be essential for insect development.

Our data also confirm that *Manduca* can feed and develop normally on *Brassica*, a non-host plant, under laboratory conditions as previously reported (Boer and Hanson, 1984). Thus this species, which is specialized on Solanaceous plants, may have the potential to switch to a new plant or extend its host-plant range. However, under field conditions, host-plant extensions have not been reported for *Manduca* larvae. One possible explanation for this discrepancy between larval performance in the lab and field might be that *Brassica* is simply not chosen for oviposition by female moths. In laboratory experiments, *Manduca* shows no oviposition preference between *Brassica oleracea* and *Nicotiana* (Späthe et al., 2013). However, since oviposition in *Manduca* is guided by olfactory stimuli, and contact chemostimulation elicits deposition of eggs (Yamamoto et al., 1969), it may be that *B. napus* is not accepted for oviposition in the field. As a result there is only a modest chance of its being accepted as a host plant, since larvae show a clear preference for the plants they have previously eaten when given a choice (Jermy et al., 1968).

Based on our RNAseq data, the GO enrichment analysis identified plant-specific overrepresented GO terms. The overrepresentation of such GO-terms related to e. g. chitin binding in larvae fed on all plants but not on artificial diet suggests that a specialized insect such as *Manduca* activates general pathways which are necessary for larval development on plants, irrespective of the plant species the larvae feed on. Chitin is an important component of the peritrophic matrix lining the midgut and is responsible for the strength, elasticity and permeability of this structure, which is an important physical and biochemical barrier (Hegedus et al., 2009; Terra, 2001). It has been hypothesized that the peritrophic matrix is involved in protecting larvae from ingested toxins (Abedi and Brown, 1961; Devenport et al., 2006). An overrepresentation of chitin-binding related genes thus might imply that these genes are another line of defense in *Manduca*

Table 4
ORs expressed in *M. sexta* larvae which have orthologues in *B. mori*, where their best ligands were identified using the expression of heterologues in frog oocytes (Tanaka et al., 2009). All listed *B. mori* ORs are also expressed in the larvae (Tanaka et al., 2009).

M. sexta OR	*B. mori* OR orthologue	Ligands for *B. mori* OR
MsexOR-30	BmOR-56	cis-jasmone
MsexOR-52	BmOR-54	cis-jasmone, Henkel 100
MsexOR-24	BmOR-42	linalool
MsexOR-18	BmOR-29	linalool, citral, linalyl aceteate

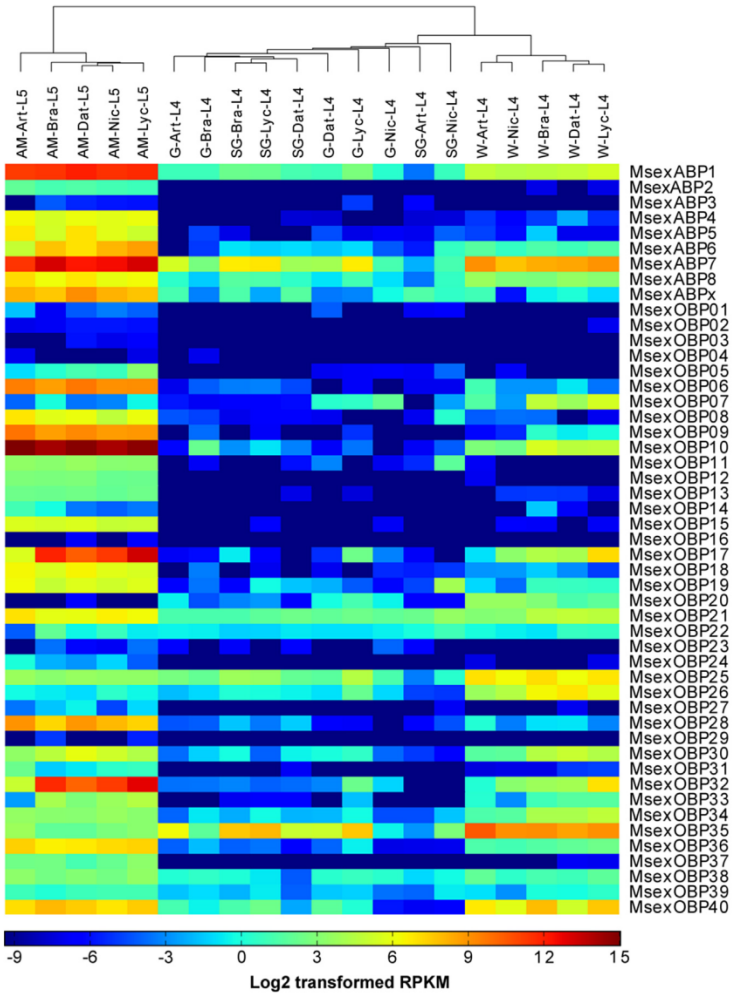


Fig. 6. Expression of odorant-binding proteins (OBP). OBP expression is highest in the antennae and maxillae samples, but there is some expression in the gut/Malpighian tubules and labial gland samples. Values are based on log2-transformed RPKM values relative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). AM: antennae and maxillae; G: guts with Malpighian tubules; SG: silk gland (labial gland); W: whole insect.

larvae, in addition to genes that assist in active detoxification as well as those that provide immunity.

Although we tested several plants to identify differentially expressed genes specific to a certain host plant the larvae fed on, it may be that some of the identified genes perform multiple roles (Vogel et al., 2014b). For example, CYP6B46, initially identified as strongly up-regulated in larvae feeding on diet containing nicotine, was proposed to be linked to nicotine-uptake or -transport processes in the larval guts of *Manduca* (Kumar et al., 2014). However, we could demonstrate that CYP6B46 is up-regulated in larvae fed on all of our tested plants (e.g. note the 19-fold change in G samples of *Brassica*-fed insects). This level of up-regulation implies a more general function for CYP6B46, instead of a clear but limited correlation with nicotine detoxification or transport as has been suggested (Kumar et al., 2014).

Most of the research in insect communication and behavioral changes has investigated adult stages, although several studies have shown that larvae can also respond to volatile cues (Del Campo, 2003; Del Campo et al., 2001; Glendinning et al., 2009). This study provides the first insight into the molecular olfactory repertoire of *Manduca* larvae. We detect the expression of 20 ORs in the AM tissue, including the putative pheromone receptor MsexOR-51 (Koenig et al., forthcoming), and MsexOR-30, an orthologue of a *B. mori* OR that is necessary for host targeting (Tanaka et al., 2009). Additionally, we report the expression of a large number of OBPs in all investigated tissues; these OBPs likely function as transporters of lipophilic substances.

We report changes in the expression of OR and OBP genes that could contribute to the response of larvae grown on different plants rather than artificial diet (Glendinning et al., 2009). If specific OBPs are missing, the response of a receptor to its ligands is decreased (Pophof, 2002). On the other hand, the majority of ORs seems to function independently of the presence of OBPs (Hallem and Carlson, 2006; Hallem et al., 2004; Swarup et al., 2011). Up- or

down-regulation of OBPs could be one mechanism larvae use to adapt the periphery of the olfactory system to specific odors. Since OBPs also interacted with gustatory receptors, the observed regulation of OBP could also influence gustation (Galindo and Smith, 2001; Jeong et al., 2013; Swarup et al., 2014). It is possible that there is a change in gene expression in other chemosensory tissues such as the labrum. We did not dissect this tissue, but its ablation is known to alter the host plant selection of *Manduca* larvae (De Boer, 1991; Del Campo et al., 2001). However, other reports emphasize the importance of the antennae and maxillae for host plant selection (De Boer, 2006, 1993; Hanson and Dethier, 1973; Waldbauer and Fraenkel, 1961). Furthermore, other chemosensory receptor types, such as gustatory receptors and ionotropic receptors, may be involved. Additionally, it is possible that the expression of other genes in the signaling cascade, rather than the expression of the receptors, is responsible for the induction events in the chemosensory tissue; or it may be that induction simply does not involve the regulation of gene expression. Therefore the effect of induction on the chemosensory tissue (Glendinning et al., 2009) may be different from the effect of changes in gene expression.

Although the direct negative effects of plant allelochemicals on larval growth and detoxification-related gene expression seem obvious, the secondary effects of plant allelochemicals on other aspects of insect physiology, such as innate immunity, have been less thoroughly investigated. In interactions among herbivorous insects, microorganisms, parasitoids and host plants, secondary compounds can have profoundly different effects on the outcome of these interactions and ultimately on insect performance and survival (Berenbaum, 1988; Mumm and Dicke, 2010; Diamond and Kingsolver, 2011; Del Campo et al., 2013). Secondary compounds can either reduce the toxicity of pathogens by reducing consumption rates of insects, or increase the toxicity of pathogens by adding extra stress to an insect's metabolism (Berenbaum, 1988; Castro et al., 2009, 2008; Navon et al., 1993). The different host plants to which *Manduca* larvae are exposed could also harbor different, possibly pathogenic microorganisms. The composition of the microbial community on both the surface and the interior of the plant leaf, locations which are known to contain diverse and dense bacterial communities, vary among conspecific plants as well as among different leaves and parts of the same plant (Meyling and Eilenberg, 2006; Monier and Lindow, 2004; Vodovar et al., 2005). Because larvae are naturally exposed to microbes via consumption, this diversity adds to the list of novel niche conditions to which herbivorous insects' immune system must adapt (Freitak et al., 2009a, 2009b, 2007). In order to maintain organismal homeostasis, insect herbivores have to respond to both the challenges posed by host plant secondary metabolites as well as to the respective microbiota they harbor. For a number of immune signaling pathway genes we observed high expression levels in the AM samples. This could indicate that the early detection of pathogens or of high microbial loads in the diet is important and starts in the mouthparts.

Consistent with the induction of immune-related genes by plant-associated microbes and/or plant secondary metabolites, most of the AMPs, although generally expressed at rather low levels, display a dynamic regulation and are differentially expressed in a diet-dependent way. Low expression levels of the AMPs compared to e.g. signaling pathway genes is plausible, since AMPs, as part of the humoral response, are regulated by and downstream of the IMD, JNK and the Toll pathway (Boutros et al., 2002; Casanova-Torres and Goodrich-Blair, 2013; Gunaratna and Jiang, 2013; Lemaitre et al., 1996, 1995). Because mounting an immune response is costly, most AMPs are expressed at measurable levels only when microbial patterns are detected and the respective signaling pathways activated. While AMP expression was overall lowest in all the tissues of larvae fed artificial diet, most AMPs also display expression differences in a complex tissue and host-plant specific way. Thus, although we could not identify a single plant with a clear differential expression pattern of immune-related genes, both host plant-associated microbiota as well as host plant chemistry does affect immune gene expression in different larval tissues in a context-dependent manner, with each host plant inducing a distinct set of immune-related genes.

Taken together, our findings demonstrate the ability of larvae of a specialized insect herbivore to efficiently use a larger repertoire of host plants than it commonly utilizes in the field. *Manduca* larvae display plastic responses at the gene expression level and offer transcriptional signatures specific to the challenges of each host- and non-host plant. In order to understand why, despite the overall positive effect of all plants tested on larval performance, *Manduca* specializes only on the nightshade family, we have to take into account the complexity of an ecosystem. *Manduca*'s preference for the nightshade family is not necessarily based on the insect's limited ability to successfully feed and develop on other plants; instead, larval preference could simply be connected to adult preference. Alternatively, larvae that are restricted from feeding extensively on these plants may receive developmental advantages in a more complex ecological context, such as host plant-dependent resistance to pathogens or parasites.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.06.001.

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Supplementary Material

Table S1: Gene names mentioned in text and figures and respective *Manduca sexta* OGS2 gene IDs.

Gene class	Msex OGS2 number	Gene name	Gene class	Msex OGS2 number	Gene name
GST	Msex2.00305	MAPEGZ1	**ABC Transporter**	Msex2.03567	A
	Msex2.00306	MAPEGZ2		Msex2.03568	A
	Msex2.00537	Delta-OLF1		Msex2.04170	A
	Msex2.00538	Delta-OLF2		Msex2.04213	D1
	Msex2.00809	Delta5		Msex2.04981	C9
	Msex2.01219	Zeta3		Msex2.05212	D1
	Msex2.02313	Theta1		Msex2.05814	H
	Msex2.02374	Omega3		Msex2.05815	H
	Msex2.02375	Omega2		Msex2.06707	C3
	Msex2.02376	Omega1		Msex2.06970	F2
	Msex2.02751	Epsilon5a		Msex2.07270	B5
	Msex2.02752	Epsilon5b		Msex2.07664	C11
	Msex2.02753	Epsilon6b		Msex2.07869	C10
	Msex2.03348	Sigma1		Msex2.08166	B4
	Msex2.03349	Sigma2		Msex2.08194	C8
	Msex2.03350	Sigma3		Msex2.08825	B6
	Msex2.03351	Sigma4b		Msex2.09106	B2
	Msex2.03352	Sigma5b		Msex2.09107	B3
	Msex2.03353	Sigma6		Msex2.09200	H
	Msex2.03355	Sigma8b		Msex2.09295	E1
	Msex2.04941	Epsilon3		Msex2.09773	B1
	Msex2.05056	Epsilon2		Msex2.09797	C6
	Msex2.08395	Epsilon8		Msex2.09851	G
	Msex2.08396	Epsilon4		Msex2.10064	B8
	Msex2.08503	Delta4		Msex2.10395	F1
	Msex2.08504	Delta3		Msex2.10847	G5
	Msex2.09785	MAPEG1		Msex2.11130	C5
	Msex2.09786	MAPEG2		Msex2.11196	G1
	Msex2.09787	MAPEG3		Msex2.11687	D2
	Msex2.11063	Omega4		Msex2.11695	G
	Msex2.11554	Zeta2		Msex2.11947	B7
	Msex2.11763	Zeta1		Msex2.12147	A3
	Msex2.11862	Delta1		Msex2.12351	A
	Msex2.11863	Delta2		Msex2.12541	G
	Msex2.15508	Epsilon6a		Msex2.12542	G4
	Msex2.15509	Epsilon7		Msex2.12678	C1
	Msex2.15513	Sigma4a		Msex2.12679	C2
	Msex2.15514	Sigma5a		Msex2.12701	G
	Msex2.15515	Sigma8a		Msex2.12844	B9
	Msex2.15517	Epsilon1		Msex2.13212	G8
ABC Transporter	Msex2.01019	white		Msex2.13266	brown
	Msex2.01020	scarlet		Msex2.13803	G2
	Msex2.01581	A5		Msex2.13929	F3
	Msex2.02789	G		Msex2.15061	C7
	Msex2.02920	G		Msex2.15073	D2
	Msex2.02921	G		Msex2.15134	C4
	Msex2.02959	G3	**UGT**	Msex2.00532	UGT42A3
	Msex2.03566	A7		Msex2.00533	UGT42B3

Gene class	Msex OGS2 number	Gene name	Gene class	Msex OGS2 number	Gene name
UGT	Msex2.00534	UGT42A4	**P450**	Msex2.01467	CYP337A
	Msex2.01742	UGT33G1		Msex2.01638	CYP4AU
	Msex2.01842	UGT39B3		Msex2.01639	CYP4AU
	Msex2.02747			Msex2.01872	CYP333A3
	Msex2.02748	UGT40A2		Msex2.01884	CYP354A5
	Msex2.04831	UGT46A5		Msex2.02491	CYP6B45
	Msex2.05185	UGT44A3		Msex2.02492	CYP6B
	Msex2.05263	UGT34A4		Msex2.02933	CYP4CG
	Msex2.06759	UGT45A1		Msex2.02935	CYP4CG
	Msex2.08124	UGT46C1		Msex2.02936	CYP4CG1
	Msex2.09140	UGT48B1		Msex2.03303	CYP332A
	Msex2.09875	UGT40E1		Msex2.03304	CYP332A
	Msex2.09876	UGT40J1p		Msex2.03305	CYP332A
	Msex2.09877	UGT40H2		Msex2.03681	CYP303A
	Msex2.09878	UGT40C1		Msex2.04085	CYP321A
	Msex2.09879	UGT40C3		Msex2.04412	CYP302A
	Msex2.09880	UGT40L2		Msex2.04540	CYP4G4
	Msex2.10200	UGT33L1		Msex2.04949	CYP4C
	Msex2.10777	UGT41C1		Msex2.04950	CYP340A
	Msex2.11004	UGT47A3		Msex2.04951	CYP340A
	Msex2.11077	UGT33H2		Msex2.04952	CYP4C
	Msex2.11078	UGT33H1		Msex2.04955	CYP4C
	Msex2.11079	UGT33E1		Msex2.05089	CYP367B
	Msex2.11080	UGT340B1		Msex2.05090	CYP4C
	Msex2.11081	UGT340A3		Msex2.05506	CYP18A
	Msex2.11082	UGT340A4		Msex2.05508	CYP18A
	Msex2.12063	UGT50A3		Msex2.05509	CYP306A
	Msex2.12523	UGT33E3		Msex2.05640	CYP4G
	Msex2.12524	UGT33C		Msex2.05641	CYP4G49
	Msex2.12525	UGT33P1		Msex2.05642	CYP4G
	Msex2.12598	UGT40A2		Msex2.05754	CYP338A
	Msex2.13845	UGT340A1		Msex2.05854	CYP49A
	Msex2.13846			Msex2.06102	CYP341A
	Msex2.13847	UGT340A2		Msex2.06392	CYP6AE
	Msex2.13848	UGT340A5p		Msex2.06452	CYP4S
	Msex2.14488	UGT50A3		Msex2.06453	CYP4S
	Msex2.15377	UGT50A3		Msex2.06640	CYP315A
	Msex2.15495	UGT33G2		Msex2.06944	CYP6AU
	Msex2.15496	UGT33G3		Msex2.06945	CYP6B
	Msex2.15497	UGT40E2		Msex2.07258	CYP49A
	Msex2.15498	UGT33E2		Msex2.07259	CYP301A
	Msex2.15529	UGT40C2		Msex2.07399	CYP305B
	Msex2.15545			Msex2.07561	CYP324A
P450	missing	CYP6B46		Msex2.07562	CYP324A
	Msex2.00093	CYP428A		Msex2.07563	CYP324A
	Msex2.01198	CYP339A		Msex2.08007	CYP304F
	Msex2.01282	CYP6A		Msex2.08108	CYP6CT
	Msex2.01466	CYP337B		Msex2.08297	CYP314A

Gene class	Msex OGS2 number	Gene name	Gene class	Msex OGS2 number	Gene name
P450	Msex2.08561	CYP6AB		Msex2.13553	CYP333B
	Msex2.08562	CYP6AB		Msex2.13589	CYP4C
	Msex2.09027	CYP6AX		Msex2.13751	CYP6BD
	Msex2.09686	CYP9AJ		Msex2.13820	CYP341B
	Msex2.09894	CYP337A		Msex2.13906	CYP6AE
	Msex2.09933	CYP341B		Msex2.14281	CYP341B
	Msex2.09934	CYP4C		Msex2.14397	CYP6BD
	Msex2.09935	CYP341B		Msex2.14567	CYP6BD
	Msex2.10033	CYP4M1		Msex2.14593	CYP340C
	Msex2.10034	CYP4M		Msex2.14600	CYP340C
	Msex2.10035	CYP4M2		Msex2.14709	CYP6BD
	Msex2.10036	CYP4M		Msex2.14778	CYP6AE
	Msex2.10215	CYP6AE31		Msex2.15113	CYP307A
	Msex2.10327	CYP15A		Msex2.15162	CYP333B
	Msex2.10760	CYP9A		Msex2.15204	CYP366D
	Msex2.10889	CYP333B		Msex2.15240	CYP6BD
	Msex2.10890	CYP333B		Msex2.15476	CYP6BD
	Msex2.10891	CYP333B	**OBP**	Msex2.00460	MsexOBP09
	Msex2.10893	CYP333B		Msex2.00461	MsexOBP10
	Msex2.10894	CYP333B11		Msex2.00462	MsexABP1
	Msex2.10895	CYP333B		Msex2.00463	MsexOBP14
	Msex2.10896	CYP333B		Msex2.00464	MsexOBP11
	Msex2.11047	CYP6AE		Msex2.00465	MSexABP2
	Msex2.11048	CYP6AE		Msex2.00466	MsexOBP12
	Msex2.11105	CYP9G		Msex2.00467	MsexABP4
	Msex2.11106	CYP9G		Msex2.00468	MsexOBP13
	Msex2.11107	CYP9G		Msex2.01454	MsexOBP15
	Msex2.11402	CYP307A		Msex2.01707	MsexOBP16
	Msex2.11700	CYP6AB13		Msex2.01708	MsexABP8
	Msex2.11774	CYP6AN		Msex2.02108	MsexABPx
	Msex2.11778	CYP9A		Msex2.02300	MsexOBP18
	Msex2.11779	CYP9A		Msex2.02656	MsexOBP20
	Msex2.11790	CYP9A		Msex2.02657	MsexOBP19
	Msex2.11791	CYP9A		Msex2.02658	MsexOBP38
	Msex2.11907	CYP341B		Msex2.02698	MsexOBP21
	Msex2.11908	CYP341B		Msex2.03248	MsexOBP22
	Msex2.11909	CYP341B		Msex2.03518	MsexOBP23
	Msex2.11957	CYP6AN		Msex2.03519	MsexOBP24
	Msex2.12312	CYP366D		Msex2.03888	MsexOBP07
	Msex2.13217	CYP6AE32		Msex2.03889	MsexABP6
	Msex2.13219	CYP6AE		Msex2.03890	MsexOBP17
	Msex2.13220	CYP6AE		Msex2.03891	MsexOBP33
	Msex2.13221	CYP6AE		Msex2.03892	MsexABP7
	Msex2.13294	CYP6AN5		Msex2.03893	MsexOBP32
	Msex2.13295	CYP6AN		Msex2.04082	MsexOBP26
	Msex2.13515	CYP4C		Msex2.05759	MsexOBP06
	Msex2.13552	CYP4L		Msex2.05760	MsexOBP03

Gene class	Msex OGS2 number	Gene name
OBP	Msex2.05761	MsexOBP01
	Msex2.05762	MsexOBP04
	Msex2.05763	MsexOBP02
	Msex2.07317	MsexOBP25
	Msex2.07430	MsexOBP05
	Msex2.07487	MsexABP5
	Msex2.08382	MsexOBP27
	Msex2.09425	MsexABP3
	Msex2.10958	MsexOBP28
	Msex2.11541	MsexOBP29
	Msex2.12548	MsexOBP39
	Msex2.13730	MsexOBP30
	Msex2.13843	MsexOBP08
	Msex2.14143	MsexOBP31
	Msex2.14192	MsexOBP34
	Msex2.14323	MsexOBP35
	Msex2.14420	MsexOBP40
	Msex2.14586	MsexOBP36
	Msex2.15032	MsexOBP37
OR	missing	MsexOR-10
	missing	MsexOR-15
	Msex2.00565	MsexOR-82
	Msex2.00624	MsexOR-38
	Msex2.00707	MsexOR-24
	Msex2.01521	MsexOR-36
	Msex2.01522	MsexOR-08
	Msex2.01523	MsexOR-33
	Msex2.01524	MsexOR-80
	Msex2.01525	MsexOR-84
	Msex2.01571	MsexOR-42
	Msex2.01618	MsexOR-66
	Msex2.02252	MsexOR-67
	Msex2.02512	MsexOR-27
	Msex2.02514	MsexOR-29
	Msex2.02515	MsexOR-68
	Msex2.02754	MsexOR-69
	Msex2.02755	MsexOR-43
	Msex2.03330	MsexOR-12
	Msex2.04326	MsexOR-62
	Msex2.04330	MsexOR-52
	Msex2.04331	MsexOR-47
	Msex2.04542	MsexOR-70
	Msex2.04689	MsexOR-71
	Msex2.04798	MsexOR-01
	Msex2.04835	MsexOR-83
	Msex2.05573	MsexOR-17
	Msex2.05711	MsexOR-13
OR	Msex2.05788	MsexOR-40
	Msex2.06100	MsexOR-72
	Msex2.06834	MsexOR-73
	Msex2.07161	MsexOR-86
	Msex2.07660	MsexOR-07
	Msex2.07682	MsexOR-06
	Msex2.07686	MsexOR-05
	Msex2.07920	MsexOR-75
	Msex2.07921	MsexOR-23
	Msex2.07922	MsexOR-41
	Msex2.08006	MsexOR-22
	Msex2.08017	MsexOR-25
	Msex2.08018	MsexOR-46
	Msex2.08303	MsexOR-64
	Msex2.08305	MsexOR-77
	Msex2.08399	MsexOR-19
	Msex2.08680	MsexOR-34
	Msex2.08681	MsexOR-16
	Msex2.08682	MsexOR-78
	Msex2.09025	MsexOR-74
	Msex2.09038	MsexOR-87
	Msex2.09279	MsexOR-26
	Msex2.09281	MsexOR-65
	Msex2.09282	MsexOR-09
	Msex2.09918	MsexOR-32
	Msex2.09919	MsexOR-31
	Msex2.09996	MsexOR-57
	Msex2.09997	MsexOR-39
	Msex2.10957	MsexOR-51
	Msex2.11073	MsexOR-30
	Msex2.11103	MsexOR-35
	Msex2.11504	MsexOR-11
	Msex2.11915	MsexOR-21
	Msex2.11916	MsexOR-20
	Msex2.12027	MsexOR-76
	Msex2.12520	MsexOR-28
	Msex2.12521	MsexOR-50
	Msex2.12779	MsexORCO
	Msex2.12902	MsexOR-88
	Msex2.13403	MsexOR-04
	Msex2.14357	MsexOR-03
	Msex2.14612	MsexOR-89
	Msex2.14943	MsexOR-18
	Msex2.14948	MsexOR-79
	Msex2.15272	MsexOR-49
n. spec.	Msex2.12103	5-TOX

Gene class	Msex OGS2 number	Gene name	Gene class	Msex OGS2 number	Gene name
AMP	Msex2.01301	Lebocin-A	**AMP**	Msex2.08545	Attacin04
	Msex2.01302	Lebocin-B		Msex2.08785	Cecropin07
	Msex2.01303	Lebocin-D		Msex2.08787	Cecropin09
	Msex2.01305	Lebocin-C		Msex2.08788	Cecropin10
	Msex2.02244	Defensin2		Msex2.09992	Lysozyme-like protein
	Msex2.04820	Lysozyme-2		Msex2.09994	Lysozyme-3
	Msex2.04821	Lysozyme-like protein		Msex2.09998	Lysozyme-like
	Msex2.04822	Lysozyme-1		Msex2.11074	Gloverin
	Msex2.04906	WAP01		Msex2.12105	Gallerimycin 2
	Msex2.04907	WAP02		Msex2.12106	Gallerimycin 3
	Msex2.05149	WAP03		Msex2.12108	Gallerimycin 1
	Msex2.05150	WAP04		Msex2.13057	AFP13
	Msex2.05151	WAP08		Msex2.13789	Cecropin01
	Msex2.05152	WAP05		Msex2.13830	AFP03
	Msex2.05153	WAP06		Msex2.13831	AFP02
	Msex2.05154	WAP07		Msex2.13832	AFP04
	Msex2.05155	WAP09		Msex2.13833	AFP05
	Msex2.05156	WAP10		Msex2.13834	AFP06
	Msex2.05157	WAP11		Msex2.13835	AFP07
	Msex2.05158	WAP12		Msex2.13836	AFP08
	Msex2.05159	WAP13		Msex2.13837	AFP09
	Msex2.05160	WAP14		Msex2.13839	AFP11
	Msex2.05161	WAP15		Msex2.13907	Cecropin02
	Msex2.05594	Moricin like peptide		Msex2.13910	Cecropin05
	Msex2.05595	Moricin 1		Msex2.13912	Cecropin06
	Msex2.05596	Moricin 2		Msex2.13920	Attacin05
	Msex2.05597	Moricin like peptide		Msex2.13921	Attacin09
	Msex2.05598	Moricin like peptide		Msex2.14460	Cecropin13
	Msex2.05599	Moricin like peptide		Msex2.14461	Cecropin14
	Msex2.06539	Defensin1		Msex2.14466	Cecropin12
	Msex2.08540	Attacin06		Msex2.14467	Cecropin11
	Msex2.08541	Attacin02		Msex2.14877	AFP14
	Msex2.08542	Attacin03		Msex2.15011	AFP01
	Msex2.08543	Attacin10		Msex2.15057	AFP12
	Msex2.08544	Attacin01		Msex2.15307	AFP10

Gene class	Msex OGS2 number	Gene name
AMP	Msex2.15523	Cecropin08
	Msex2.15547	Cecropin03
	Msex2.15548	Cecropin04
Effector genes	Msex2.09885	PPO
	Msex2.11367	PPO
IMD	Msex2.01596	IAP1 similar
	Msex2.01715	IBM1 (IAP antagonist)
	Msex2.02602	TAK1
	Msex2.05476	death domain protein
	Msex2.05545	TAB
	Msex2.05604	IAP2
	Msex2.05607	IAP1
	Msex2.06959	Dnr1
JAKSTAT	Msex2.07815	Domeless
	Msex2.08880	hopscotch
	Msex2.10552	STAT
	Msex2.11519	SOCS
	Msex2.12153	STAT
JNK	Msex2.01399	Hemolin-LP2
	Msex2.02705	Hem
	Msex2.05111	Jun- / JNK
	Msex2.09018	Hemolin-LP1
	Msex2.09332	Hem
	Msex2.09807	Hemolin
	Msex2.09858	Fos
MAPK	Msex2.04292	ERK
	Msex2.04304	MAPK
	Msex2.07041	GTPase-activating protein
	Msex2.07813	MKK3
	Msex2.08319	MAPK2
	Msex2.08712	MAPKKK
	Msex2.09629	MAPK
	Msex2.10253	MEKK1
MBP	Msex2.06179	MBP
microbial recognition	Msex2.07575	Nimrod
microbial recognition	Msex2.07576	Nimrod-B
	Msex2.07580	Nimrod-LI
	Msex2.12427	TEP1
	Msex2.13097	TEP2
PORC	Msex2.12453	PORC
PRR	Msex2.03605	PGRP-03
	Msex2.03606	PGRP-04
	Msex2.03607	PGRP-02
	Msex2.09015	PGRP-08
	Msex2.09398	PGRP-11
PRR	Msex2.13829	BGRP3
	Msex2.14130	BGRP2
	Msex2.14131	BGRP
	Msex2.15530	PGRP-05
	Msex2.10091	PGRP-09
	Msex2.10092	PGRP-01
	Msex2.10093	PGRP-13
	Msex2.10096	PGRP-06
	Msex2.10097	PGRP-07
	Msex2.11527	PGRP-12
	Msex2.11767	PGRP-11
	Msex2.13760	BGRP1
Toll	Msex2.02793	cactus
	Msex2.02909	Spz1
	Msex2.03391	Spz4
	Msex2.04007	Toll-like
	Msex2.04433	Spz3
	Msex2.07379	Spz6
	Msex2.08663	Tube
	Msex2.09986	Spz2
	Msex2.11498	dif
	Msex2.11500	Dorsal
	Msex2.11537	Toll interacting protein
	Msex2.12529	Spz5
	Msex2.13883	Toll-like
	Msex2.14106	Pelle
Binding and removal of free ions	Msex2.02637	Transferrin 1
	Msex2.10790	Transferrin2, partial
	Msex2.10792	Transferrin 2
	Msex2.10793	Transferrin2, partial
	Msex2.12754	Transferrin 3

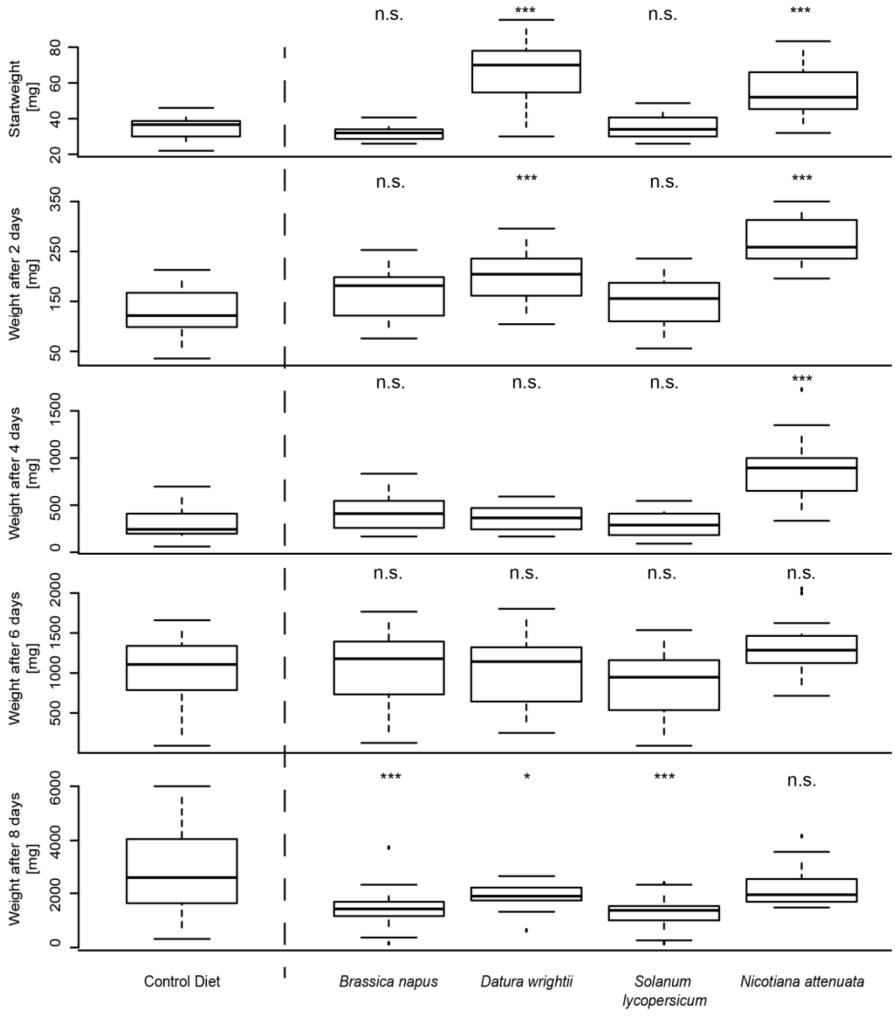

Figure S1: Larval development on each host or non-host plant for five consecutive time points (T1-T5). P-value: n.s. > 0.05, * < 0.05, ** < 0.01, * < 0.001 (ANOVA); N = 20 / plant or diet, respectively.**

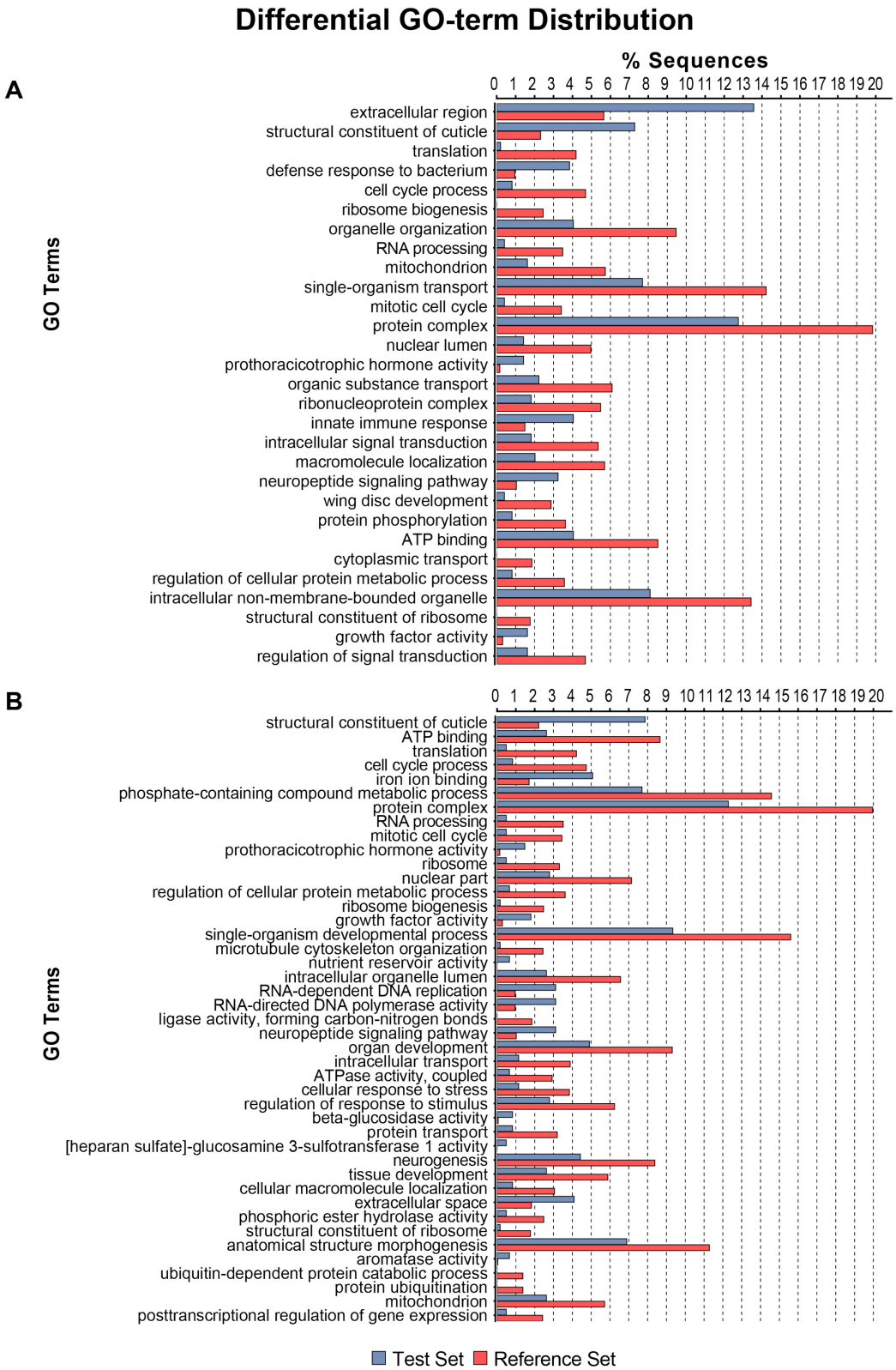

Figure S2: Differential GO term representation between larvae fed on control diet or plant material. Differences are shown as the percentage of sequences associated with a specific GO category in the reference set (*Manduca sexta* OGS2) against the test set (number of differentially expressed genes with a fold change of at least 8 between control and treatment) using Fisher's exact test (Blast2GO) A) *Brassica napus* B) *Datura wrightii.*

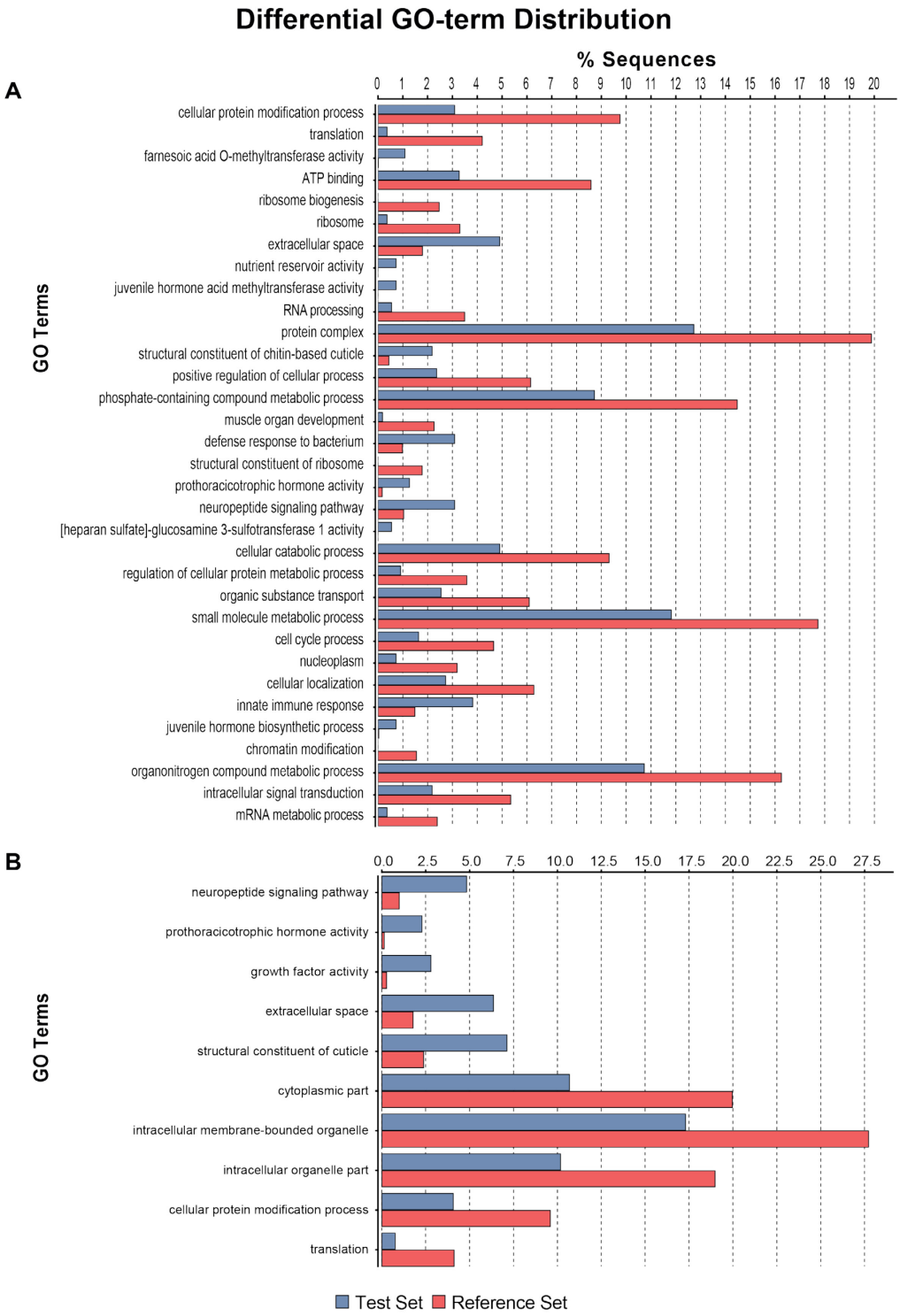

Figure S3: Differential GO term representation between larvae fed on control diet or plant material. Differences are shown as the percentage of sequences associated with a specific GO category in the reference set (*Manduca sexta* OGS2) against the test set (number of differentially expressed genes with a fold change of at least 8 between control and treatment) using Fisher's exact test (Blast2GO) A) *Solanum lycopersicum* B) *Nicotiana attenuata.*

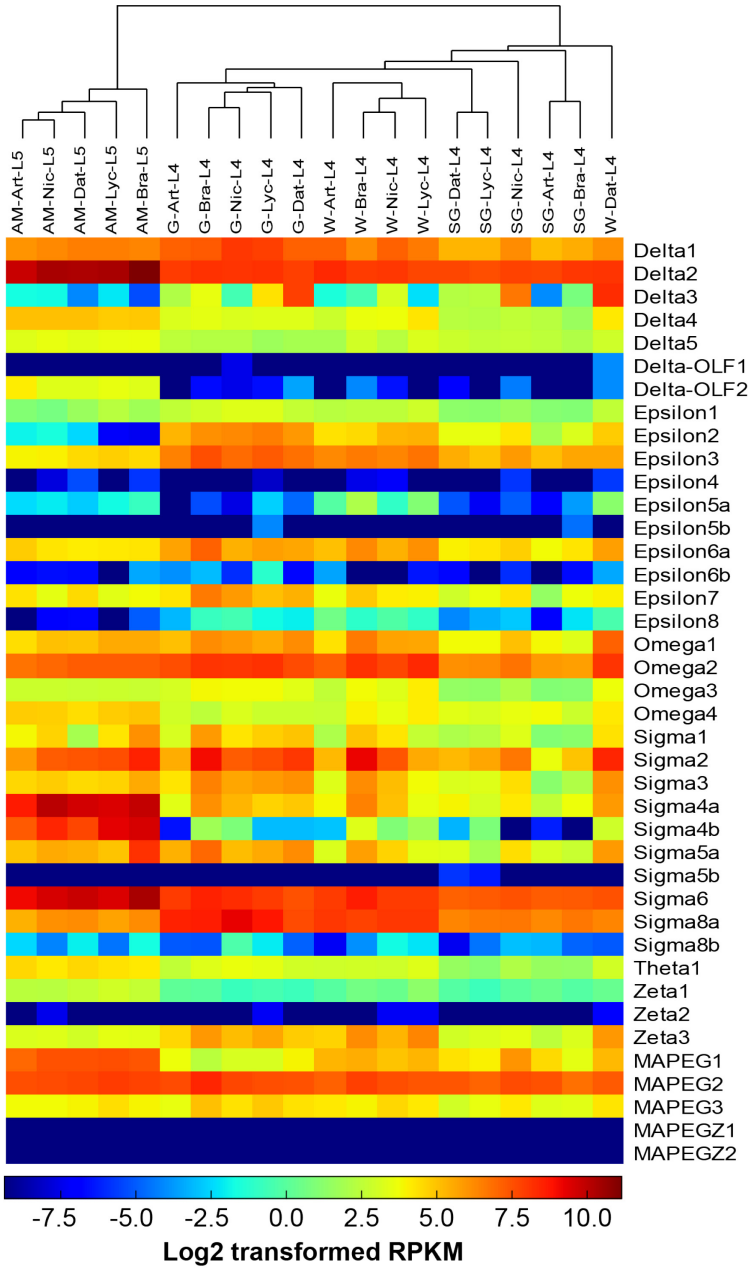

Figure S4: Expression of genes belonging to the glutathione S-transferase (GST) family. Expression levels are dependent on the food source and the tissue type. Gene expression levels in the different tissues are based on log2-transformed RPKM values and responses are expressed relative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). AM: antennae and maxillae; G: gut with Malpighian tubules; SG: silk gland (labial gland); W: whole insect

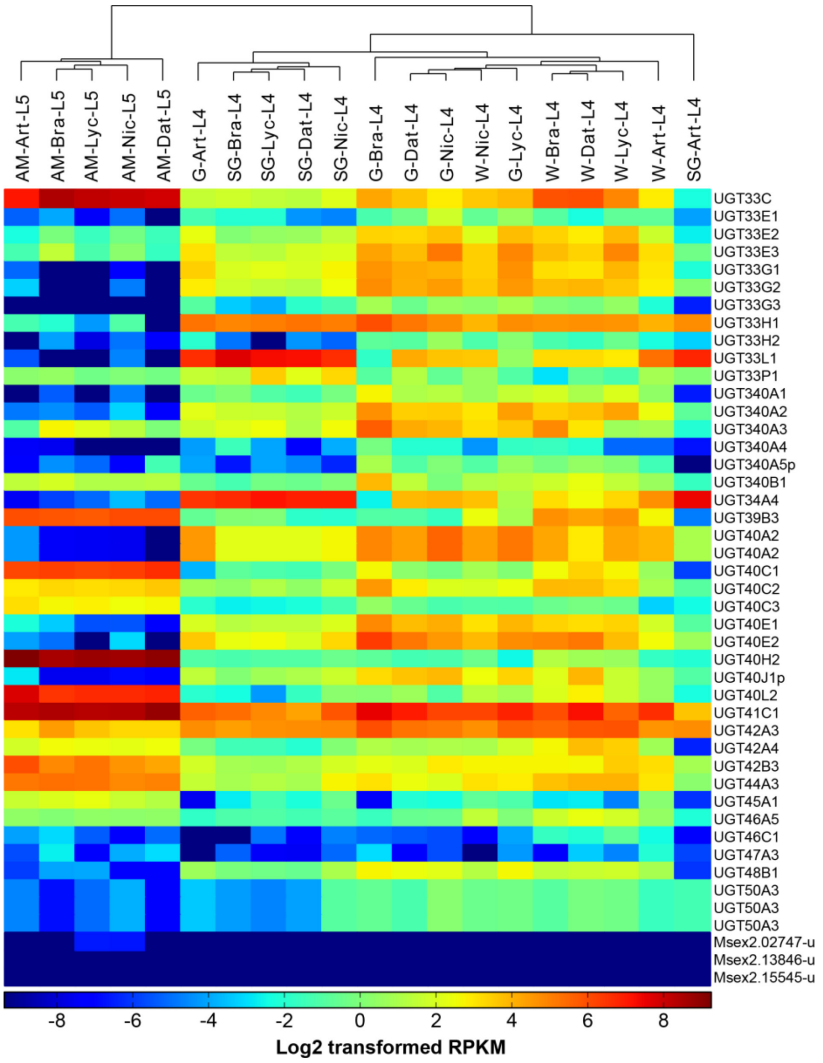

Figure S5: Expression patterns of UDP-glycosyltransferase (UGT) genes. Gene expression levels in the different tissues are based on log2-transformed RPKM values and responses are expressed relative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). AM: antennae and maxillae; G: guts with Malpighian tubules; SG: silk gland (labial gland); W: whole insect

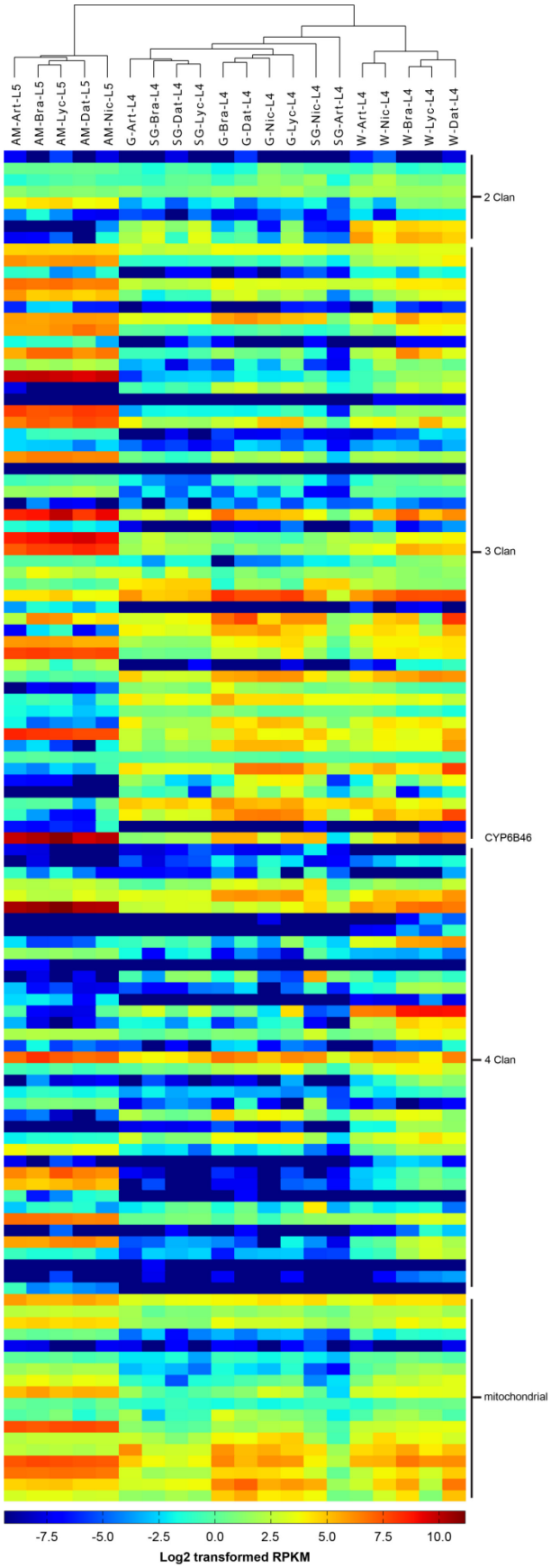
AM-Art-L5
AM-Bra-L5
AM-Lyc-L5
AM-Dat-L5
AM-Nic-L5
G-Art-L4
SG-Bra-L4
SG-Dat-L4
SG-Lyc-L4
G-Bra-L4
G-Dat-L4
G-Nic-L4
G-Lyc-L4
SG-Nic-L4
SG-Art-L4
W-Art-L4
W-Nic-L4
W-Bra-L4
W-Lyc-L4
W-Dat-L4
2 Clan
3 Clan
CYP6B46
4 Clan
mitochondrial
-7.5
-5.0
-2.5
0.0
2.5
5.0
7.5
10.0
Log2 transformed RPKM

Figure S6: Differential expression of Cytochromes P450 in *Manduca* larvae feeding on different plants. Values are based on log2-transformed RPKM values relative to the median intensity of all contigs (blue = down-regulation; red = up-regulation). AM: antennae and maxillae; G: guts with Malpighian tubules; SG: silk gland (labial gland); W: whole insect

3. Unpublished data

3.1 Identification of ABC transporters in three lepidopteran species

The genome data presented in this chapter constitute the basis for the transcriptome studies (**Chapter 2.2** and **Chapter 2.3**).

3.1.1 Introduction

ATP-binding cassette (ABC) transporters form a heterogenic multigene family, organized in different subfamilies. The members differ according to their function, domain organization and size.[8] Even though ABC transporters occur in all organisms from prokaryotes to eukaryotes, most of the knowledge derives from research in vertebrates and bacteria.[9, 17, 18, 36] To build up the foundation for future studies in insects, ABC transporters were annotated in three lepidopteran species (*Helicoverpa armigera*, *Spodoptera frugiperda* and *Manduca sexta*).

3.1.2 Materials and Methods

Gene identification and curation

The *Manduca* and the *Helicoverpa* official gene set 2 (OGS2)-predicted genes were annotated as described in **Chapter 2.2** and **2.3**. The Manduca OGS2 can be accessed from https://i5k.nal.usda.gov/.

In summary, the ABC transporter reference sequences obtained from *Bombyx mori* [38, 39], were used to perform a tblastN against the respective genome with an E-value cut-off $\leq 1e^{-4}$. By using Web Apollo, the best hit per transcript was curated in regards to intron-exon-borders, missing exons and frameshifts. The membrane topology of the protein sequences was predicted with Phobius (http://phobius.sbc.su.se/) and TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The *S. frugiperda* genes were annotated using the same procedure.

Lepidopteran ABC transporter phylogeny

The phylogenetic analysis comprised in total 244 amino acid sequences from seven lepidopteran species (*H. armigera*, *M. sexta*, *S. frugiperda*, *Danaus plexippus*, *Heliothis virescens*, *Heliothis subflexa* and *Trichoplusia ni*). The sequences for *D. plexippus*, *H. virescens*, *H. subflexa* and *T. ni* were obtained by screening the NCBI database. All sequences were cut down to contain only the most conserved region, which is the nucleotide binding domain (NBD). Full ABC transporters were cut between the two NBDs and both halves were separately included (**Figure 1.1 A**). The respective halves are marked in the phylogeny (N-terminal: ".1", black; C-terminal: ".2", white). MEGA5 was used to perform a MUSCLE Alignment with the default settings. The evolutionary history was inferred by using the Maximum Likelihood method based on the WAG model plus a Gamma distribution (the determined best model) combined with a bootstrap analysis (1000 replicates). All the positions with 0% site coverage were eliminated. The tree was drawn to scale, with branch lengths representing the number of substitutions per site.

Table 3.1| Comparison of gene numbers of ABC transporters in nine arthropod species (*Bombyx mori*, *Helicoverpa armigera*, *Manduca sexta*, *Spodoptera frugiperda*, *Drosophila melanogaster*, *Tribolium castaneum*, *Chrysomela populi*, *Tetranychus urticae*, *Daphnia pulex*) and *Homo sapiens*.[8, 18, 24, 34-40]

Subfamily	*B. mori* [a]	*H. armigera* [b]	*M. sexta* [b]	*S. frugiperda* [bc]	*D. melanogaster*	*T. castaneum*	*C. populi*	*T. urticae*	*D. pulex*	*H. sapiens* [d]
A	9	7*	8		10	10	5	9	4	12
B	9	11*	9*	8*	10	6	8	24	7	11
C	15	11*	11*	11*	12	35	29	39	7	12
D	2	2*	2		2	2	2	2	3	4
E	1	1*	1		1	1	1	1	1	1
F	3	3*	3		3	3	3	3	4	3
G	13	16*	16		15	13	14	23	24	5
H	3	3*	3		3	3	3	22	15	-
Total	**55**	**54**	**53**		**56**	**73**	**65**	**103**	**65**	**48**

[a] Numbers represent the highest identified number of genes for the respective subfamily.[39, 40]

[b] Gene numbers are taken from the respective genome annotation project.

[c] Missing gene numbers denote that the data is incomplete.

[d] *Homo sapiens* do not possess ABC transporters of the subfamily H.

*** The genes were annotated by Anne Bretschneider.**

3.1.3 Results and Discussion

The annotation of ABC transporters in three lepidopteran species revealed both conservation and expansion of gene numbers in the eight ABC subfamilies (**Table 3.1**). Among insects, irrespective of the organism, the gene numbers in the ABCD, ABCF and ABCH subfamily are absolutely conserved. Moreover a single gene was identified in the ABCE subfamily, which persisted in all arthropod species and *Homo sapiens*. ABCE is a key player in transcription and translation and is involved in shuttling proteins between the nucleus and the cytoplasm.[27] Since these fundamental cell processes are highly conserved across kingdoms, there was selection pressure to keep this gene but most likely not to accumulate duplicates in the ABCE subfamily.

Differences regarding the gene number were discovered in the B, C and G subfamilies. All three are associated with detoxification processes.[39] One might argue that gene number in these subfamilies in arthropods correlates with the feeding strategy (specialist or generalist). Since specialists are more likely to encounter a range of rather uniform host plant-derived compounds, including a small set of secondary metabolites, they evolved efficient and constitutive defense mechanisms.[50] In contrast, generalists possess a wider range of counteradaptations, since they encounter a broad range of different secondary metabolites.[59] As a consequence one would expect to identify more detoxification-related ABC transporters in a generalist than in a specialist. However, although this holds true for the ABCB and G, this correlation is not reflected by the number of genes of the ABCC subfamily. *Bombyx mori* is a specialist, feeding exclusively on mulberry, but it possesses more ABCCs than the generalists *H. armigera* and *S. frugiperda*. [220, 221] On the other hand *B. mori* possesses less ABCG genes than *H. armigera*. As a result of species wise differing numbers of the detoxification related ABCs (subfamilies B, C and G) the generalist *H. armigera* possesses a similar total amount of corresponding genes in comparison to the specialist *B. mori* (38 and 37 respectively). However, there is one support for this "feeding-strategy dependent ABC transporter gene expansion" hypothesis: the spider mite *Tetranychus urticae*. The spider mite feeds on more than 1.100 plants and it was argued that the massive amount of ABC transporter genes correlates with the amount of host plants, yet it was not shown.[35] However, the number of genes does not allow for conclusions regarding the function of the gene. Therefore functional characterizations are needed to understand why some subfamilies expand in certain species and why other subfamilies do not.

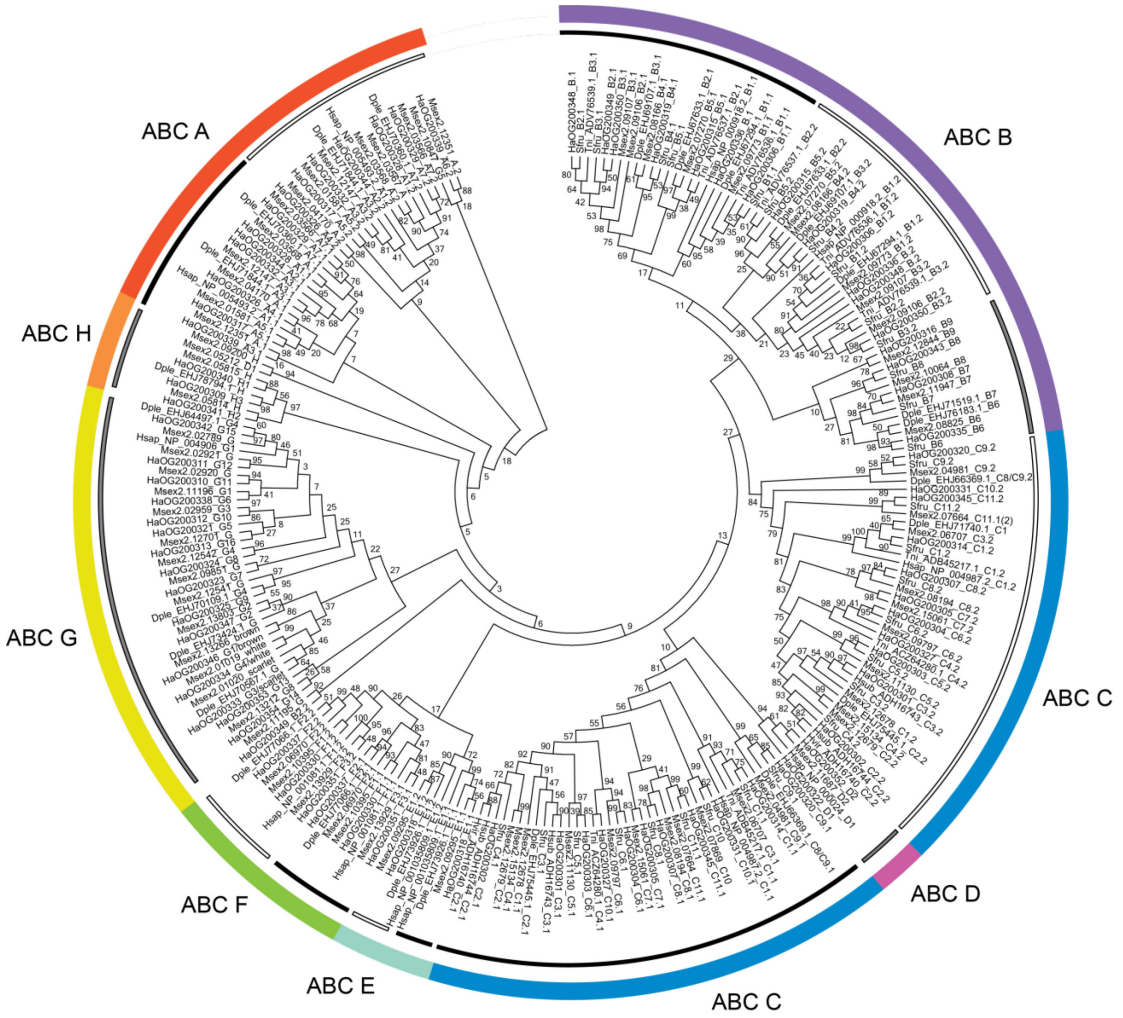

Figure 3.1| Phylogenetic tree of ABC transporters in seven lepidopteran species. The Maximum Likelihood phylogeny is based on the WAG model with a Gamma distribution in combination with a bootstrap analysis (1000 replicates). The phylogenetic analysis was conducted in MEGA5. To confer subfamily affiliation, a member of each subfamily of *Homo sapiens* was included. N- (black) and C-terminal (white) sequence parts are denoted. Half-transporters are shown in grey. Dple, *Danaus plexippus*; Ha, *Helicoverpa armigera*; Hvir, *Heliothis virescens*; Hsap, *Homo sapiens*; Hsub, *Heliothis subflexa*; Msex, *Manduca sexta*; Sfru, *Spodoptera frugiperda*; Tni, *Trichoplusia ni.*

In **Figure 3.1** the phylogenetic relationships of all the eight subfamilies of ABC transporters of seven lepidopteran species are displayed. The Maximum Likelihood phylogeny supported the subfamily affiliation of the annotated genes. Except for three cases (HaOG200349_B2.2, Msex2.05212_D1, Msex2.10847_G5), each sequence clustered according to the blast and multiple sequence alignment predictions. This implies that all subfamilies were already present in a common lepidopteran ancestor. The node support was very low in a few cases (< 20), which is however in agreement with other phylogenetic reconstructions of ABC transporters and is in general most

likely caused by the heterogeneity of this multigene family.[8, 24, 39] The subfamily A is the only one that is not monophyletic and the two subfamilies ABCE and ABCF, whose members lack transmembrane domains, cluster together.

ABC transporters possess either one (half-transporter) or two (full-transporter) nucleotide binding domains (NBD) (**Figure 1.1A**), which are important for binding two ATP molecules during the transport mechanism.[14] The subfamilies ABCA, ABCC, ABCE and ABCF only contain full-transporters, whereas the ABCD, ABCG and ABCH subfamilies are exclusively composed of half-transporters. Interestingly, the ABCB subfamily was the only one that was identified to contain both, full- and half-transporters. For all full transporters the N- and C-terminal NBDs form distinct branches but cluster subfamily wise, yet there is one peculiarity. The subfamilies B, C and D are closest relatives and form a single clade. Surprisingly, the C-terminal part of the ABCC subfamily clusters on the same branch like the entire ABCB subfamily (**Figure 3.1**). These findings have been also made in *H. sapiens*.[8] This implicates that the ABCB subfamily and the ABCC C-terminal halves share a common ancestor. Beyond that, the N-terminal NBD of the ABCC subfamily forms a clade with the ABCD subfamily, which also suggests common ancestry.

We could identify orthologous genes in *H. armigera*, *M. sexta* and *S. frugiperda* across all ABC subfamilies. Interestingly, when orthologs were identified in all three species, *H. armigera* and *S. frugiperda* clustered together (one exception: ABCB7). This reflects the species phylogeny, since both belong to the Noctuidae. However, only two subfamilies (B and C) were annotated in *S. frugiperda* so far.

In summary, the multigene family of ABC transporters shows different duplication events: i) domain-duplication (full-and half-transporters), ii) gene-duplication and iii) lineage-specific gene-duplication. These events led to the diversification of ABC transporters and a species-specific set of ABCs in addition to a well conserved core composition.

4. General Discussion

Agricultural insect pests may not only encounter plant defense mechanisms, such as secondary metabolites, but also insecticides. Hence, they need to be adapted to different chemical challenges. This thesis investigates the role of ATP-binding cassette (ABC) transporters in herbivorous Lepidoptera adapting to these challenges. The basis for this was provided by the annotation of the ABC transporter multigene family in three lepidopteran species (**Chapter 3**). Following this, the transcriptional signatures were investigated during insect development (egg to adult) and in different larval tissues (**Chapter 2.2**). To examine the role of ABC transporters in detoxification, the impact of host plant and non-host plant derived secondary metabolites on ABC transporter gene expression was explored in a generalist (**Chapter 2.2**) and a specialist lepidopteran species using RNAseq (**Chapter 2.3**). To complete the picture about ABC transporters, one gene was heterologously expressed and its involvement in insecticide resistance characterized (**Chapter 2.1**). This thesis contributes to our understanding of the different functions of ABC transporters in lepidopteran insects and to elucidating their role in insect detoxification pathways.

4.1 Genome-wide ABC transporter identification

ABC transporters have been identified in all organisms from bacteria to human [8], yet the information in insects is scarce. Therefore, ABC transporter genes were annotated in the genomes of the lepidopteran species *Helicoverpa armigera*, *Spodoptera frugiperda* and *Manduca sexta*. All three species have a comparable number of ABC transporter genes, similar to the number identified in *Bombyx mori* (**Chapter 3: Table 3.1**). Furthermore, a phylogenetic analysis revealed orthologous genes in all three species, whereby the two noctuid moths (*H. armigera* and *S. frugiperda*) were more closely related in comparison to the sphingid species (*M. sexta*) (based on node support), which is consistent with evolutionary distance. ABC transporters share high sequence similarity, especially in the nucleotide binding domain and not only among lepidopteran species. And since they can be found in all organisms, it has been proposed that they have a common evolutionary origin.[222] Furthermore, it can be concluded that both gene as well as domain duplication led to the diversification of this gene family. However, the driving

forces for this diversification are not yet known. In herbivorous insects, it may be that environmental conditions and species ecology, such as feeding range and especially the host plant defense mechanisms, might have caused this diversification (**Figure 4.1**), as it has been proposed before in *Tetranychus urticae*.[35] Evidence for this hypothesis can be found in overlapping substrate ranges among members of the same ABC subfamily as well as across different subfamilies.[223] However, it also suggests that the neofunctionalization among ABC transporters is not yet complete.

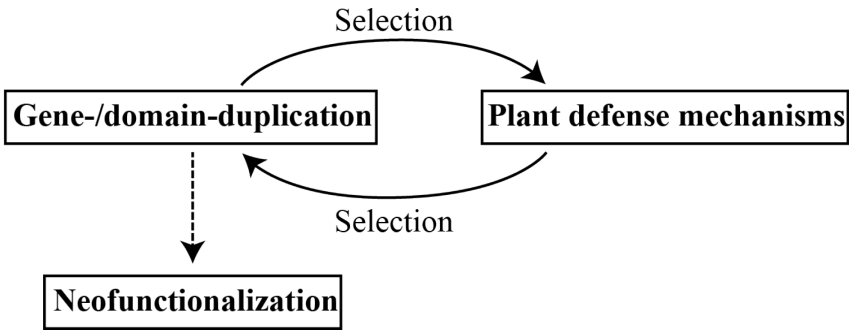

Figure 4.1| Proposed rapid evolution of detoxification-related ABC transporter genes in herbivorous insects.

The RNAseq data from *H. armigera* and *M. sexta* revealed that all identified genes are transcribed and are therefore functional genes, and not just artefacts (**Chapter 2.2** and **Chapter 2.3**). Furthermore, the ABC genes show a complex pattern of developmental- and tissue-specific expression, as also shown in *H. armigera* (**Chapter 2.2**). Similarly, the mosquito *Anopheles gambiae* displays a developmental regulation of detoxification-related genes, such as cytochrome P450s, carboxylesterases and glutathione S-transferases (GSTs), in larvae, pupae and adults.[218] Furthermore, ABC transporter genes were specifically expressed in different life stages in the spider mite *T. urticae*.[35] These findings can be explained by different nutrient requirements as well as diets consumed during an insect's life span, e.g. a leaf feeding lepidopteran larvae may require and encounter different nutrients than a pollen feeding adult.

4.2 Generalist and specialist insect herbivores overlap in their adaptation to plant secondary metabolites

Plants produce secondary metabolites as a defense mechanism against herbivorous insects. However, insects may respond by avoiding feeding on respective tissues or by adapting gene expression.[72, 109, 224] In order to examine the latter, the transcriptome of a

generalist (*H. armigera*) (**Chapter 2.2**) and a specialist lepidopteran species (*M. sexta*) (**Chapter 2.3**) were analyzed in relation to feeding on host plants and non-host plants or secondary metabolites (**Figure 4.2**), respectively. *Helicoverpa* displayed a general detoxification response as was expected for a highly polyphagous herbivore. *Manduca* larvae on the other hand exhibited host plant and non-host plant specific ABC transporter expression signatures. The results show that ABC transporter gene expression is affected by the feeding on different plants and secondary metabolites. This suggests their involvement in detoxification pathways, either by directly transporting the xenobiotic or by taking part in a more complex stress response.

4.2.1 Adaptive gene regulation and detoxification of plant secondary metabolites

Specialist and generalist insect herbivores are considered to differ regarding their range of biochemical defense strategies.[225] Specialists can maximize their development by investing in a few efficient and constitutive defense mechanisms, since they are very likely to encounter a range of rather uniform secondary metabolites.[50] In contrast, generalists possess many different biochemical defense strategies at the cost of lower feeding success, in order to cope with a wider range of plant defense mechanisms.[59] However, the information on global gene responses of herbivores to plant toxins is scarce.[87]

A first step in order to disentangle the insect adaptation mechanisms is to identify candidate genes by analyzing transcriptome data.[72, 87, 109] This approach may reveal a connection of a gene with the ability to feed on plants and thus supporting the development. For example, the transfer to a different host plant caused differential gene expression in 28 ABC transporters (103 were identified in total) in the polyphagous spider mite *T. urticae*.[35] Especially the subfamilies C and G, both related to detoxification processes, and H were affected.[35, 39] Similar results were obtained in the present studies (**Chapter 2.2** and **Chapter 2.3**). Interestingly, *H. armigera* (Noctuidea) and *M. sexta* (Sphingidae) overlap partially in their transcriptional response towards dietary stress, even though they show different feeding strategies; one is a generalist and the other a specialist. Both species upregulate ABCC1 (named ABCC3 in *M. sexta* (**Chapter 3: Figure 3.1**)) when feeding on tomatine or tomato, and ABCB3 when larvae are exposed to nicotine or tobacco. Noctuidea and Sphingidae are distantly

related [226], yet they potentially exploit similar mechanisms to adapt to plant defensive chemistry by upregulating orthologous genes. The fact that both studies independently identified ABCB3 and ABCC1, suggests the involvement of both proteins in the detoxification of these secondary metabolites. However, it remains to be shown whether these are indeed direct responses to the plant defensive compounds or simply stress-related and undirected.[87]

Overlapping adaptive gene regulation in generalists and specialists has been shown before, not only for ABC transporters.[75] For example, the lepidopteran species *Helicoverpa assulta* (specialist) and *H. armigera* (generalist) are closely related, and both tolerate high concentrations of capsaicin, an alkaloid present in the plant genus *Capsicum*. In order to detoxify this host plant-derived compound, both species utilize UGTs.[50, 75]

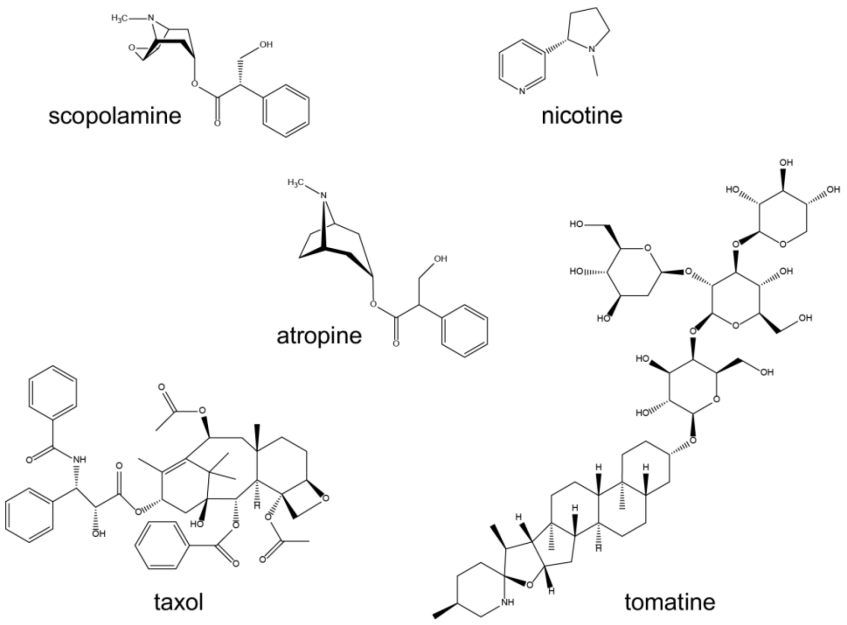

Figure 4.2| Chemical structures of plant secondary metabolites, which were fed to *H. armigera* in the transcriptome study (Chapter 2.2) and which are present in the host plants of *M. sexta* (except taxol, Chapter 2.3).[227]

The detoxification of nicotine by an ABC transporter has been proposed in several studies before, though with unclear results.[83, 84] While one study proposes that ABCB1 is responsible for nicotine efflux from the Malpighian tubules in *M. sexta*, another study claims that efflux may be due to an ABC transporter, but not ABCB1. The present results identified an ABCB3 upregulation upon nicotine treatment in two distantly related species in two independent studies. ABCB1 and ABCB3 are paralogous genes and belong to the same subfamily.[36] Interestingly, ABC transporter proteins are also

hypothesized to take part in the nicotine membrane transport in plants, which could underline that nicotine is detoxified through export.[85] Such overlap of ABC transporter function between different kingdoms has been shown before between plants, human and yeast.[228] Additional studies on ABCB3 might resolve its biochemical function in insects, and possibly its importance for nicotine detoxification. Govind et al. (2010) showed that the larval feeding of *Manduca* on wildtype and transgenic *Nicotiana attenuata* affected ABC transporter expression in a diet-specific way.[225] Interestingly, when plant nicotine production was silenced, *M. sexta* reacted with the downregulation of ABC genes.[225] This further suggests that ABC transporters are involved in nicotine detoxification.

The upregulation of ABC transporters in response to toxic stress is a reliable indicator to identify candidates.[205] For example, cytotoxic resistant human cancer cells overexpress ABCB1 (also known as Pgp) and ABCB2 (also known as TAP1).[8, 229, 230] Both genes were later shown to detoxify cytotoxic drugs through export, therefore conferring multidrug resistance.[23, 230, 231]

ABC transporters form a diverse family, exemplified for example by the broad spectrum of recognized substrates.[7] Many ABC transporters, especially the bacterial importers, interact with a single substrate or a single substrate class.[9, 232] On the other hand, there are the so called "multidrug pumps", e.g. Pgp and LmrA, which transport plenty of compounds.[19, 233, 234] Although there has been a vast amount of studies on identifying substrates for multidrug transporters and extracting their physicochemical properties to summarize general substrate properties [235-237], this did not result in the possibility to predict ABC transporter substrates. Therefore, simply based on the chemical structure it is not possible to conclude whether the secondary metabolites fed to *Helicoverpa* and *Manduca* larvae in the transcriptome analyses, are substrates for ABC transporters and therefore transported (**Figure 4.2**). Furthermore, several factors have been proposed which influence the sensitivity of an ABC transporter. Mutations in the transporter can massively alter substrate translocation. Different mutations in the maltose transporter MalK in *Escherichia coli* and *Salmonella typhimurium* showed for example ATP binding but no transport.[238] In addition, membrane lipids can influence ABC transporter sensitivity as shown for Pgp, which preferentially works in the vicinity of phosphatidylethanolamines.[239]

Apart from pure compounds, a few ABC transporters show affinity for xenobiotic conjugates. A known example is the ABCC subfamily and its interaction with glutathione-conjugated substrates generated by GSTs.[240, 241] The *M. sexta* RNAseq data revealed GST gene upregulations in response to plant feeding, yet the response was not specific (**Chapter 2.3**). Similar results were found in the aphid *Myzus persicae* and in *H. armigera*.[69, 72] In order to identify such potential gene interactions in the present RNAseq data, a weighted correlation network analysis (WGCNA) could be performed. This method allows the identification of clusters of highly correlated genes, for example in pathways, and linking them to a phenotype.[242, 243] Nevertheless, the structure of the transcriptome data in **Chapter 2.2** and **Chapter 2.3** does not allow performing such an analysis.

4.2.2 Further approaches to elucidate secondary metabolite detoxification by ABC transporters

It cannot be ruled out that the ABC transporters, which displayed differential gene expression patterns in response to different treatments, perform functions apart from the ones mentioned. Therefore, further studies are required.

A reasonable approach would be the heterologous expression of candidates, for example in cell lines. These can be analyzed regarding their transport properties, such as export of xenobiotics, thus verifying that the tested compound is a substrate for the respective ABC transporter. For example a study in the leaf beetle *Chysomela populi* expressed the ABC transporter CpMRP in *Xenopus laevis* oocytes.[42] Hereby, it was directly shown that CpMRP transports salicin, a secondary metabolite present in the host plant of *C. populi*. Another way to prove the status of a substrate is to measure the accumulation of the compound in so called “inside-out-vesicles”.[244] By “flipping” the membrane from the inside to the outside an exporter is turned into an “importer”. This will lead to accumulation of a xenobiotic in the vesicle, if it is a substrate. The compound could then be measured for example by high-performance liquid chromatography (HPLC).[245]

Keeping in mind the bigger picture, the resolution of a detoxification pathway may be accomplished through the analysis of metabolites. The analysis of the feces, the gut content or the hemolymph of an insect after feeding on a certain secondary metabolite, could provide valuable information on the metabolization of the xenobiotic before the excretion, as well as potential conjugation and subsequent transport processes.

4.3 ABC transporters and their role in insecticide resistance

ABC transporters have been linked to confer resistance to synthetic insecticides.[19] For example ABCB1 confers resistance to Fenvalerate and Cypermethrin in *H. armigera*, as well as Thiodicarb resistance in *Heliothis virescens*.[44-48] Furthermore, ABC transporter gene expression was shown to be different in two multiresistant *T. urticae* strains when compared to a susceptible wildtype strain, suggesting an involvement of these transporters in insecticide resistance processes as well.[246]

However, ABC transporters may also function as receptors for *Bacillus thuringiensis* derived insecticidal toxins (Bt). This gram-positive bacterium produces insecticidal proteins, which interact with receptors in the insect gut membrane. Therefore, two putative receptors (HevABCC2 and HevCaLP) from the agricultural pest *H. virescens* were stably expressed in non-lytic clonal Sf9 insect cell lines (**Chapter 2.1**). Mortality assays, binding studies and time lapse recordings identified the ABC transporter HevABCC2 as the main target for the Cry1A toxins (Cry1Aa, Cry1Ab and Cry1Ac) and underlined its central role in the mode of action in *H. virescens*. Furthermore, the results illustrate an enhancing effect of HevCaLP on the intoxication process.

4.3.1 The ABCC2 transporter in *Heliothis virescens* functions as a receptor to Bt toxins

Different models have been proposed for the Bt mode of action throughout the last years.[143, 157, 159, 161] Based on the current results, a new model for the mode of action for Cry1A toxins can be proposed (**Figure 4.3**). The toxin monomers bind to HevCaLP, which leads to the clipping of the α1 helix. Subsequently the monomers oligomerize in solution, forming a pre-pore which binds to HevABCC2. Eventually, the oligomer is released into the cellular membrane, where it forms a pore. This pore formation leads to the loss of the membrane function and ultimately to insect death. However, the presence of HevCaLP enhances this process.

The results with cells only expressing HevABCC2 suggest that there is a limit to pore formation, because the cells only swell to a certain extent. An explanation might be that all HevABCC2 transporters are “saturated” with bound Cry1A toxin oligomers. However, the co-expression of HevCaLP removes this limit, because those cells show a faster and stronger swelling. These results suggest that HevCaLP catalyzes the insertion of additional pre-pore structures by removing inserted pores from their association with

the HevABCC2 target. Interestingly, both proteins need to be expressed in the same cell; otherwise they are not close enough for such an interaction.

This new model combines aspects of the other proposed mode of actions. The "classical model" already incorporated the toxin activation and pore formation, yet it did not identify specific receptors.[143, 157] The "sequential binding" model however, proposes a so called "ping-pong" mechanism of the activated toxin between an aminopeptidase N (APN) and CaLP. [147, 148, 152, 159] Based on the current results it is not possible to conclude whether there is a "ping-pong" mechanism, yet the results point to an interaction of the Cry toxin with two different receptors (HevABCC2 and HevCaLP). Interestingly, the expression of HevABCC2 and HevCaLP in cell lines was sufficient to cause high mortality upon Cry1A treatment, despite the absence of other putative receptors, such as APNs and alkaline phosphatases (ALP), which was verified by qRT-PCR. The results suggest that proteins from the APN and ALP gene families are not the initial targets for Cry1A toxins, yet their involvement cannot be excluded.

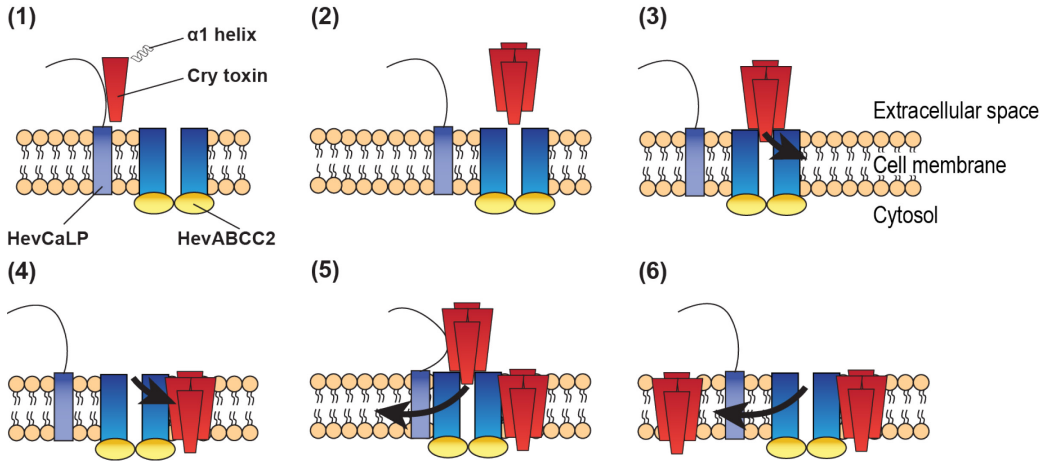

Figure 4.3| Proposed Cry1A toxin mode of action in *Heliothis virescens*. The Cry1A toxin monomers bind to HevCaLP and the α1 helix is cleaved of (1). The monomers oligomerize and form a pre-pore in solution (2). The pre-pore binds to HevABCC2 (3) and is inserted into the membrane (4). Furthermore, the pre-pore binds to the HevCaLP (5), which pulls the oligomer into the cell membrane (6); thus, causing an enhancement of the pore formation.

Different studies have connected ABC transporters to Bt resistance before, and thus implied their importance for the mode of action of Cry toxins. Especially ABCC2 has been shown to be a target for Bt toxins in different lepidopteran species, apart from the present study. When ABCC2 from *Bombyx mori* (BmABCC2) was transiently expressed in Sf9 cells, it was shown that it serves as a functional receptor to Cry1Ab.[186]

Furthermore, Cry1A resistant strains of *Trichoplusia ni*, *Helicoverpa armigera* and *B. mori* harbored mutations in the ABCC2 transporter gene which were in complete linkage with the resistance.[181-183] ABCC3, which is closely related to ABCC2, was suggested to be a receptor for Cry1Ca in *Spodoptera exigua* larvae.[149] Furthermore, based on genetic markers Bt resistance in the western corn rootworm *Diabrotica virgifera virgifera* was linked to an ABC transporter as well.[247] Interestingly, ABC transporters from other subfamilies may also act as targets for Bt toxins. Just recently, mutations in the ABCA2 transporter were linked to Cry2Ab resistance in *H. armigera* individuals.[203] All these studies underline the crucial role of the ABC transporter gene family for the mode of action of Bt toxins. However, this is the first study to stably express an ABC transporter to characterize its function in the Bt mode of action.

So far, information on how the toxin and HevABCC2 interact is limited. However, the interaction is hypothesized to be of a transient nature due to the ATP-switch mechanism of the ABC transporter.[248] This means that the toxin is either pushed into the cell membrane (irreversibly), where it forms a pore, or it is pushed back into the gut lumen (reversibly). A XenTari®-resistant *Spodoptera exigua* population (Xen-R) harbors a 246 bp deletion in the second nucleotide binding domain (NBD) of the ABCC2 transporter.[149] As both NBDs are involved in the ATP binding during the transport mechanism, this deletion will also affect the functionalization of the transporter.[14] The Xen-R population showed a lower irreversible binding, implying a lower membrane insertion of the Bt toxin. Together with the hypothesized transient interaction of the toxin with the ABC transporter, this would suggest that the toxin binds in the actual channel, which is formed by the ABC transporter for shuttling compounds. However, due to the deletion this mechanism does not function anymore and the channel is not formed. To underline this assumption, Baxter et al. showed that a 30 bp deletion resulted in the loss of the 12th transmembrane domain of the ABC transporter in *Plutella xylostella*.[182] This eventually caused the C-terminal NBD to be on the extracellular side. Again this would affect the overall ABC transporter conformation and thus also toxin binding, which was shown to be absent in Bt resistant *P. xylostella* larvae.[249] These examples point towards the Cry1A toxin binding in the channel formed by the ABC transporter, yet further evidence is needed (**Chapter 4.3.2**). In summary, the current

findings illustrate that ABC transporters function as targets for Bt toxins and thus should be put at the center of attention when deciphering the Bt mode of action.

4.3.2 Further steps to resolve the Cry1A toxin mode of action

Even though HevABCC2 has been shown to be the main target for Cry1A toxins in *H. virescens,* further experiments are conceivable, especially to resolve unknown aspects about the mode of action.

In order, to obtain a better understanding of the receptor-toxin interaction, especially the identification of the ABC transporter regions involved in the binding of the Cry1A toxin, the ATP-switch mechanism that drives the substrate transport may be utilized (**Figure 1.1** and **Figure 4.4 B**). By treating cells which stably express HevABCC2 with substrates or inhibitors, the transporter conformation can be controlled. A substrate would induce the transport mechanism and therefore the ABC transporter would constantly switch between the open and the closed dimer position. Thus the Cry1A toxin would be allowed to bind only for a short amount of time, if it binds into the channel formed by the ABC transporter (**Figure 1.1 B**). Therefore, it would be expected that less toxin would be bound, if a substrate is present. This could be visualized by Western blot, since Cry1A toxins are proteins. In addition, using inhibitors would underline these findings, since the inhibitor would "fixate" the transporter in one position. Nevertheless, when the toxin binds to one of the extracellular loops of the ABC transporter, the amount of bound toxin would be unaffected by the usage of substrates and inhibitors. However, the drawback is that these experiments require a substrate or inhibitor, respectively, while exactly this kind of information is rarely available for insect ABC transporters.

Furthermore, mutagenesis studies could be performed. These experiments could further resolve Cry toxin binding by identifying amino acid regions which take part in the toxin-transporter interaction. Different mutation sites have already been linked to Bt resistance (**Figure 4.4 A**). For example, a study on ABCC2 in *Bombyx mori* revealed that a tyrosine mutation at position 234 significantly reduced the intoxication of Cry1Ab and Cry1Ac.[186]

Regarding other receptors, it might be worthwhile to investigate the roles of ALPs and APNs. These membrane bound proteins have been proposed to function as receptors for Cry1A toxins as well.[147, 148, 152] They perform different cellular functions, such as cell

adhesion and peptide metabolism, or they have a hydrolytic function.[153, 154] Co-expression of these two genes in different combinations with HevABCC2 and HevCaLP could illustrate their potential involvement in the Cry1A toxin mode of action. A conceivable result could be an enhancement of the intoxication, since an interaction between the toxin and the ALP and APN has already been shown.[147, 159]

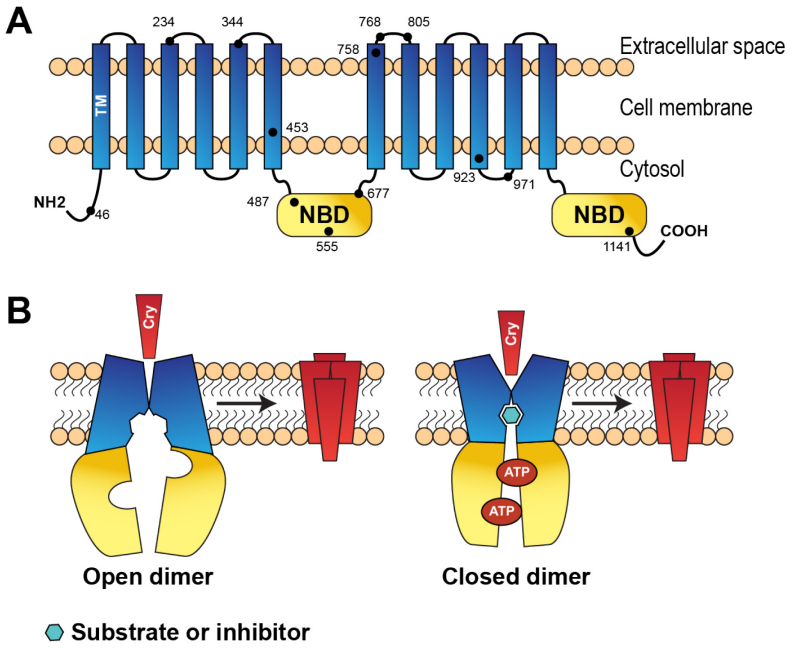

Figure 4.4| Future approaches to resolve the Cry1A toxin mode of action. A) Observed mutation sites in ABCC2 in *Bombyx mori*. Numbers represent amino acids (adapted from [186]). B) Binding of Cry1A toxins to different conformations of the ABC transporter, which may be determined by the use of a substrate or an inhibitor. TM, transmembrane domain; NBD, nucleotide binding domain; Cry, Cry1A toxin.

Most of the research on Bt has focused on identifying putative receptors and resistance mechanisms, rather than revealing the mode of action in a step by step fashion. Both, the "classical model" and the "sequential binding model", propose a membrane insertion and pore formation of the activated Cry toxin.[143] Nevertheless, this has not been fully elucidated. The pore structure and the actual mechanism of membrane insertion may be visualized by cryoelectron microscopy. This method was successfully applied to elucidate the pore structure formed by tripartite ABC-type toxin complexes produced by the insect pathogenic bacterium *Photorhabdus luminescens*.[250] Both modes of action have in common that they take place at the membrane surface and that the toxin initially binds to a receptor. Furthermore, this experiment would resolve whether the toxin-transporter-interaction is transient, like it is hypothesized.[248] If this is the case,

one would expect to find a connection of toxin and ABC transporter after membrane insertion. The stable clonal cell lines expressing HevABCC2 and HevCaLP (**Chapter 2.1**) would allow performing this experiment.

4.4 The role of ABC transporters in insect pest species and future prospects

Herbivorous insect pests face a complex set of environmental challenges, such as pathogens and predators, but also dietary toxin stress. While ABC transporter proteins are involved in different life aspects, such as development, eye pigmentation, and predator defense mechanisms [34, 42, 43], most importantly they are involved in the transport and detoxification of xenobiotics, thereby causing resistance.

Until today, there has not been a case of field-evolved resistance to synthetic insecticides which is caused by ABC transporters. However, the ability to cause multidrug resistance may soon be a threat to agriculture. Current studies have shown the potential of the ABCB1 transporter to shuttle insecticides, fungicides, herbicides, alkaloids and antibiotics.[19, 44-48]

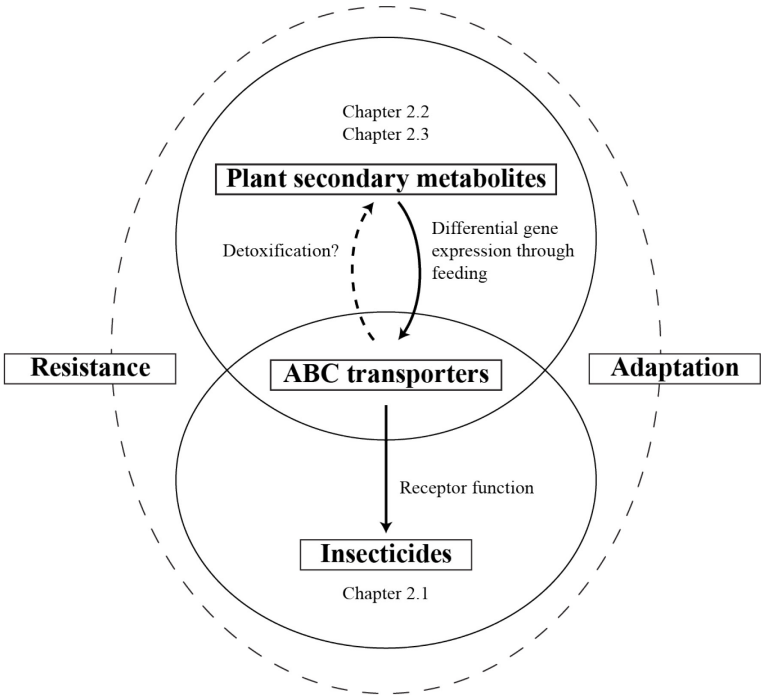

Figure 4.5| ABC transporters in insect detoxification pathways.

One way of bypassing this threat has been the simultaneous use of combinations of different insecticides.[251, 252] Even though this might aim at different target sites, it may further select for cross-resistance and should therefore not be seen as a reliable approach. A similar approach was chosen for the use of Bt-based insecticides.[168, 253, 254]
The general understanding of the Bt mode of action in insect pests could help improving Bt insecticides, for example through the identification of new receptors. Some Bt toxins have already been modified in such a way that they show higher binding affinity or do not depend on a receptor for pore formation anymore.[255] The advantage of this approach was supposed to be the delay of resistance; however, first cases of resistance to these new Bt toxins were already documented, for example in the western corn rootworm *D. v. virgifera*.[137]
This thesis contributes to the current knowledge on ABC transporters, especially extending the knowledge on this multigene family in lepidopteran insects. The present results identified ABC transporters to be associated with adaptation mechanisms in generalist and specialist insect herbivores (**Figure 4.5**). These results provide fundamental insights into the detoxification mechanism of insects focusing on insect-plant-adaptations and furthermore may lead to the improvement of insecticides and future pest management strategies.

5. Summary

Almost half of all insects in the world feed on plants; however plants exploit defense mechanisms of both physical and chemical nature, such as secondary metabolites. Insects on the other hand have developed ways to circumvent these defense barriers, for example by detoxifying the secondary metabolites through metabolization and excretion. Furthermore, herbivorous insects encounter additional challenges, such as insecticides. A suitable instrument to overcome both obstacles may be the multigene family of ATP-binding cassette (ABC) transporters, which comprises eight subfamilies encoding for transmembrane proteins that actively export a wide range of substrates out of the cell. These proteins have been associated with various functions in eukaryotes and prokaryotes; however information in insects is scarce. Therefore, the aim of this thesis was to identify ABC transporters in lepidopteran herbivores and to investigate their role in insect detoxification pathways in relation to insect-plant adaptation mechanisms as well as their role in insecticide intoxication.

(i) The annotation of ABC transporters revealed the conservation of gene numbers across the two noctuid species *Helicoverpa armigera* and *Spodoptera frugiperda* as well as the sphingid moth *Manduca sexta*. The numbers were comparable to that of the lepidopteran model organism *Bombyx mori*. Orthologous genes were identified in *H. armigera*, *M. sexta*, and *S. frugiperda* across all ABC subfamilies, suggesting that the lepidopteran ancestor already possessed genes in all the eight ABC subfamilies. Furthermore, the gene clusters reflected the species phylogeny, showing that the genes of the two noctuid moths were closer related to each other than to these of the sphingid moth. In summary, gene-, domain- and lineage-specific gene duplication led to the ABC transporter diversification in lepidopteran insects.

(ii) The generalist *H. armigera* was used to investigate how polyphagous insect herbivores detoxify plant secondary metabolites from host plants (atropine and scopolamine, nicotine, and tomatine) and non-host plants (taxol) and how ABC transporters are putatively involved. A replicated RNAseq experiment revealed specific expression in different life stages as well as different tissues for genes

encoding ABC transporters; for example detoxification related ABC transporters were primarily expressed in the larval gut and the Malpighian tubules. A developmental assay with various secondary metabolite-containing diets showed that the larval development was similar on the atropine-scopolamine-containing diet and the tomatine-containing diet. The latter induced a treatment-specific upregulation of five ABC genes in the gut. Feeding either on nicotine or taxol caused similar detrimental effects on the larvae. Remarkably, both xenobiotics induced the upregulation of the same ABC transporters. These results demonstrate that the polyphagous herbivore *H. armigera* exploits general detoxification mechanisms against these secondary metabolites present in the different host plants.

(iii) In contrast, specialist insect herbivores are expected to have evolved specific adaptation mechanisms to the major defense barriers of their narrow range of host plants. In order to study the effect of host plants and a non-host plant on the larvae of *Manduca sexta*, which is a specialist on solanaceous plants, a replicated RNAseq experiment in combination with a larval developmental assay was conducted. Larvae developed fastest on *Nicotiana attenuata*, yet no significant difference in performance was observed for *Manduca* larvae fed on the other solanaceous plants or the non-host plant *Brassica napus*. The transcriptome data revealed specific transcriptional signatures towards the challenges of each host plant and non-host plant; especially members of detoxification-related gene families such as glutathione S-transferases (GST), cytochrome P450s and ABC transporters were upregulated. These results show that *M. sexta* has evolved specific adaptation mechanisms to different host plants and that this specialist is able to efficiently use a broader repertoire of host plants than it actually utilizes in the field.

(iv) *Heliothis virescens* (Lepidoptera) is a polyphagous insect pest species. In order to diminish its populations, insecticidal toxins from the gram positive bacterium *Bacillus thuringiensis* (Bt, Cry toxins) are used, which interact with receptors in the midgut membrane. Two putative receptors, the ABC transporter HevABCC2 and the cadherin-like protein HevCaLP, were heterologously expressed in an insect-derived cell line (Sf9). Four clonal stable expressing cell lines (i) wild type, ii) HevCaLP, iii) HevABCC2, iv) HevCaLP and HevABCC2) were produced, which

enabled the extensive analysis of the Bt mode of action without potential side effects usually caused by the transfection using baculoviruses. qRT-PCR confirmed that other putative receptors were not endogenously expressed in Sf9 cells. Interestingly, three Cry1A toxins (Cry1Aa, Cry1Ab, Cry1Ac) caused similar effects. The double transfected cells swelled and died, whereas the HevABCC2-expressing cells just showed swelling. The wild type and HevCaLP-only expressing cells on the other hand were unaffected. Cell-based binding assays revealed that the toxin-receptor interaction is specific. Eventually, these results were confirmed by performing an *in vivo* toxicity assay with *H. virescens* larvae. These experiments identified HevABCC2 as the central target for Bt toxins in the insect midgut membrane and demonstrate that HevCaLP increases Cry1A toxicity. Consequently, the long lasting debate about the role of ABC transporters in the Bt mode of action could be clarified by deduction of a new model, which is based on these complex investigations.

This thesis contributes to the current knowledge on ABC transporters, but especially extending the knowledge on this multigene family in lepidopteran insects. The role of ABC transporters in detoxification processes was characterized on a genomic as well as a transcriptomic level. These results provide fundamental insights into the detoxification mechanism of insects focusing on insect-plant-adaptations. In addition, one candidate gene was functionally characterized regarding its role in the Bt mode of action. This illustrates that ABC transporters are not only involved in detoxification mechanisms but also support intoxications. Beyond, this knowledge will support the future development of new pest management strategies in agriculture.

6. Zusammenfassung

Nahezu die Hälfte aller Insekten weltweit ernährt sich von Pflanzenmaterial. Allerdings besitzen Pflanzen die vielfältigsten Verteidigungsmechanismen, sowohl physische als auch chemische, wie zum Beispiel Sekundärmetaboliten. Insekten wiederum haben Wege entwickelt diese Verteidigungsmechanismen zu umgehen, indem sie beispielsweise diese Sekundärmetaboliten durch Metabolisierung und Exkretion detoxifizieren. Zusätzlich zu diesen bestehenden Herausforderungen müssen herbivore Insekten oft auch Insektiziden widerstehen. Eine Möglichkeit hierfür stellt eine Multigenfamilie dar, welche für Transporter mit ATP-bindender Kassette (ABC) codiert. Diese Transmembranproteine, welche sich in acht Unterfamilien aufteilen, sind aktiv für den Export von Substraten verantwortlich (u.a. Xenobiotika). Die Funktionen dieser Proteine in Eukaryoten und Prokaryoten sind hinreichend bekannt, jedoch ist das Wissen über die Funktionen in Insekten noch sehr begrenzt. Deshalb war das Ziel dieser Arbeit zunächst ABC Transporter in verschiedenen Lepidoptera-Arten zu identifizieren und sie im Hinblick auf Insekten-Pflanzen-Interaktionen und Insektizid-Resistenzen funktionell zu charakterisieren.

(i) Die Anzahl der ABC Transporter Gene in den drei Lepidoptera-Arten *Helicoverpa armigera* (Noctuidae), *Spodoptera frugiperda* (Noctuidae) und *Manduca sexta* (Sphingidae) ist konserviert und vergleichbar mit der Anzahl in dem Modelorganismus *Bombyx mori*. In allen drei Arten wurden orthologe Gene identifiziert, was vermuten lässt, dass der gemeinsame Vorfahre bereits Gene aus allen acht ABC-Unterfamilien besessen hat. Darüber hinaus reflektieren die Gencluster die Verwandtschaftsbeziehungen der Arten untereinander. So sind zum Beispiel die Gene der Noctuidae *H. armigera* und *S. frugiperda* näher miteinander verwandt als mit den Genen von *M. sexta*. Insgesamt führten Gen-, Domän- und artspezifische Genduplikationen zur Auffächerung der ABC Transporter in Lepidoptera.

(ii) Zur Identifizierung wie polyphage Insekten die Sekundärmetaboliten ihrer Wirtspflanzen mithilfe von ABC Transportern detoxifizieren, wurden Raupen von

H. armigera mit verschiedenen Sekundärmateboliten aus Wirtspflanzen (Nicotin, Atropin, Scopolamin, Tomatin) und einer Nichtwirtspflanze (Taxol) gefüttert und das Transkriptom mittels RNAseq analysiert. Erstmalig konnte in einem Nachtfalter gezeigt werden, dass ABC Transporter sowohl Gewebe-spezifisch als auch Entwicklungsstadien-spezifisch exprimiert werden. Zum Beispiel werden im Darm und den Malpighischen Gefäßen primär ABC Transporter exprimiert, die an Detoxifizierungsmechanismen beteiligt sind, wie es bereits in anderen Organismen gezeigt wurde. Die Fraßstudie zeigte, dass sich *Helicoverpa*-Raupen auf Atropin-Scopolamin-Futter und Tomatin-Futter ähnlich entwickeln. Letzteres induzierte die spezifische Expression von fünf ABC Genen. Das Fressen auf Nicotin oder Taxol verursachte ähnliche Entwicklungsverzögerungen in den Raupen und induzierte die Hochregulierung der gleichen ABC Transporter Gene. Insgesamt veranschaulichen diese Ergebnisse, dass der Generalist *H. armigera* allgemeine biochemische Mechanismen verwendet, um die verschiedenen Sekundär-metaboliten in den einzelnen Wirtspflanzen zu detoxifizieren.

(iii) Im Gegensatz dazu sind Spezialisten nur einer begrenzteren Anzahl von Sekundärmetaboliten ausgesetzt und besitzen daher spezifischere Detoxifizierungsmechanismen. Um diese biochemischen Anpasssungs-mechanismen eines Spezialisten an verschiedene Pflanzen zu untersuchen, wurde der Einfluss von Wirts- (Solanaceae) und Nichtwirtspflanzen (*Brassica napus*) auf das Transkiptom und die Entwicklung von *Manduca sexta*-Raupen analysiert. Auf der Wirtspflanze *Nicotiana attenuata* entwickelten sich die Raupen am schnellsten. Interessanterweise wurde zwischen den anderen Wirtpflanzen und *B. napus* kein Unterschied in Bezug auf die Entwicklung festgestellt. Die RNAseq Ergebnisse zeigten, dass *M. sexta* spezifisch auf die unterschiedlichen Pflanzen reagiert. Insbesondere Gene, die an der Detoxifizierung von Xenobiotika beteiligt sind, wurden hochreguliert, zum Beispiel Glutathion-S-Transferasen, Cytochrome P450s und ABC Transporter. Diese Arbeit zeigt, dass *M. sexta* sich spezifisch an verschiedene Wirtspflanzen und die jeweiligen Abwehrmechanismen angepasst hat. Darüber hinaus ist diese Spezies in der Lage, ein breiteres Wirtspflanzenspektrum zu nutzen als sie im Feld tatsächlich verwendet.

(iv) *Heliothis virescens* (Lepidoptera) ist ein landwirtschaftlicher Schädling, dessen massive Vermehrung durch den Gebrauch von Insektiziden aus dem Gram-positiven Bakterium *Bacillus thuringiensis* (Bt, Cry Toxine) eingedämmt wird. Die Besonderheit dieser Insektizide besteht darin, dass es Proteine sind, welche mit Rezeptoren in der Darmmembran von Insekten interagieren. Zwei dieser mutmaßlichen Rezeptoren, der ABC Transporter HevABCC2 und das Cadherin-ähnliche Protein HevCaLP, wurden heterolog in Insektenzellen (Sf9) exprimiert und vier stabile klonale Zelllinien selektiert (i) Wildtyp, ii) HevCaLP, iii) HevABCC2, iv) HevCaLP und HevABCC2). Dies ermöglichte umfangreiche Experimente ohne eventuelle Nebeneffekte, die bei der Transfizierung mit Baculoviren auftreten können. Mittels qRT-PCR konnte gezeigt werden, dass keine weiteren mutmaßlichen Rezeptoren endogen in Sf9-Zellen exprimiert werden. Die drei Cry-Toxine Cry1Aa, Cry1Ab und Cry1Ac hatten ähnliche Effekte. Die co-transfizierten Zellen schwollen an und starben nach der Behandlung mit den Cry–Toxinen ab. Zellen, die HevABCC2 allein exprimierten, schwollen an; während Wildtyp Zellen und Zellen, die nur HevCaLP exprimierten, keine Veränderungen zeigten. Bindungsstudien zeigten zudem, dass die Toxin-Rezeptor-Interaktion spezifisch ist. Zusätzlich wurden die Ergebnisse durch eine Toxizitätsstudie in *H. virescens*-Raupen bestätigt. Diese Ergebnisse identifizieren HevABCC2 als den primären Rezeptor für die Bt-Toxine in der Darmmembran von Insekten und zeigen zudem, dass HevCaLP den Wirkungsmechanismus beschleunigt. Anhand dieser umfangreichen Ergebnisse wurde ein neues Modell für den Wirkungsmechanismus von Bt-Toxinen entwickelt.

Diese Arbeit trägt zum aktuellen Wissen über ABC Transporter bei, erweitert jedoch insbesondere das Wissen über diese Multigenfamilie in Lepidoptera. Die Rolle von ABC Transportern in Detoxifizierungsmechanismen in verschiedenen Nachtfaltern wurde sowohl auf genomischer als auch auf transkriptionaler Ebene charakterisiert. Diese Ergebnisse liefern wichtige Rückschlüsse auf Detoxifizierungsmechanismen in Insekten, insbesondere für Insekten-Pflanzen–Interaktionen. Zusätzlich konnte gezeigt werden, dass ABC-Transporter als Rezeptoren für Insektizide aus dem Bakterium *B. thuringiensis* fungieren und von großer Bedeutung für deren Wirkungsmechanismus sind. Mit diesem Wissen lassen sich neue Ansatzpunkte für das Schädlingsmanagement in der Landwirtschaft entwickeln.

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Curriculum Vitae

Anne Bretschneider

29. April 1986 in Berlin
married (maiden name: Karpinski)

Vitae

since 09/2011	**PhD**, Friedrich-Schiller-University Jena
11/ 2011	**Diploma**, Biology, Friedrich-Schiller-University Jena
08/2002-06/2003	**Exchange Student at a Highschool**, Bedford, TX, USA

Education

since 09/2011	**PhD Student**, Max Planck Institute for Chemical Ecology, Jena Supervisor: Prof. Dr. David G. Heckel ABC transporters in insect detoxification pathways
07/2010-08/2011	**Diploma Thesis**, Max Planck Institute for Chemical Ecology, Jena Supervisor: Dr. Astrid T. Groot Behavioral and genetic analysis of the level of host specialization in a generalist moth and its impact on sexual communication
10/2006-08/2011	**Diploma Study**, Biology, Friedrich-Schiller-University Jena
06/2006	**Secondary School**, Abitur, Nelly-Sachs Oberschule, Berlin

Research Experience

Dissertation, Max Planck Institute for Chemical Ecology, Jena

- Investigating the role of HevABCC2, HevCaLP for the mode of action of Bt Cry1A toxins in *Heliothis virescens*
- Analyzing the role of ABC transporters for the detoxification of plant secondary metabolites in *Helicoverpa armigera* using RNAseq
- Transcriptome analysis of *Manduca sexta* (Focus: Immune system, Detoxification, Olfaction)
- Annotation of ABC transporters in the genomes of *H. armigera*, *M. sexta* and *Spodoptera frugiperda*

Diploma Thesis, Max Planck Institute for Chemical Ecology, Jena

- Analysis of Host plant specialization in *H. virescens* by using biochemical (GC-Analysis of Pheromones), behavioral and genetic methods (QTL)

Certificates

- § 15 Gentechnik-Sicherheitsverordnung (GenTSV)
- Einführung in GxPs, mit Schwerpunkt auf GMP und GLP
- Leadership- Projektmanagement- and Team-Kurs

Social Commitment

since 2013	**PhD Representative** MPI for Chemical Ecology, Jena
since 2013	**PhD Representative** IMPRS Jena
2008-2010	**Fachschaftsrat**, Faculty for Biology and Pharmacy, Friedrich-Schiller-University Jena

Update: 10.12.2015

Publications, Presentations and Awards

Publications

Koenig, Christopher; Bretschneider, Anne; Heckel, David G.; Grosse-Wilde, Ewald; Hansson, Bill S.; Vogel, Heiko (2015). The plastic response of *Manduca sexta* to host and non-host plants. *Insect biochemistry and molecular biology*, *63*, 72-85.

Karpinski, Anne; Haenniger, Sabine; Schöfl, Gerhard; Heckel, David G.; Groot, Astrid T. (2014). Host plant specialization in the generalist moth *Heliothis virescens* and the role of egg imprinting. *Evolutionary Ecology*, *28* (6), 1075-1093.

Submitted

Bretschneider, Anne; Heckel, David G.; Pauchet, Yannick (2015). Three toxins, two receptors, one mechanism: Mode of action of Cry1A toxins from *Bacillus thuringiensis* in *Heliothis virescens*. Submission to *PLOS Pathogens*

Bretschneider, Anne; Heckel, David G.; Vogel, Heiko (2015). Know your ABCs: Characterization and gene expression dynamics of ABC transporters in the polyphagous herbivore *Helicoverpa armigera.* Submission to *Insect Biochemistry and Molecular Biology*

In preparation

Kanost, Michael; Bretschneider, Anne; et al. . Multifaceted Biological Insights from a Draft Genome Sequence of a Lepidopteran Insect, *Manduca sexta*. Submission to *Genome Biology*

Awards

Travel Award of the Bacteria Division: *Cry1A toxins cause rapid cell lysis in a clonal stable non-lytic expression system, expressing ABC-C2 and Cadherin*. International Congress on Invertebrate Pathology and Microbial Control and the 48th Annual Meeting of the Society for Invertebrate Pathology, Vancouver, CA

Presentations

Talks

Bretschneider, Anne (2015). *Cry1A toxins cause rapid cell lysis in a clonal stable non-lytic expression system, expressing ABC-C2 and cadherin*. International Congress on Invertebrate Pathology and Microbial Control and the 48th Annual Meeting of the Society for Invertebrate Pathology, Society for Invertebrate Pathology, Vancouver, CA

Karpinski, Anne; Vogel, Heiko; Heckel, David G. (2014). *How to cope with your environment - The role of ABC transporters in detoxification processes*. 9th International Workshop on Molecular Biology and Genetics of the Lepidoptera, Kolympari, Crete, GR

Karpinski, Anne (2014). *The ABC of drugs: Differential Gene Expression through Host Plant Toxins*. Talk presented at 13th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Poster

Karpinski, Anne; Pauchet, Yannick; Vogel, Heiko; Heckel, David G. (2014). Role of ABC-C2 in the interactions of *Heliothis virescens* with its host plants and Bt toxins. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

Karpinski, Anne; Pauchet, Yannick; Vogel, Heiko; Heckel, David G. (2014). Role of ABC-C2 in the interactions of *Heliothis virescens* with its host plants and Bt toxins. Poster presented at 47th Annual Meeting of the Society for Invertebrate Pathology, Mainz, DE

Karpinski, Anne; Pauchet, Yannick; Vogel, Heiko; Heckel, David G. (2014). Getting rid of Toxins - ABC transporters in Insect Detoxification Pathways. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE

Karpinski, Anne (2014). The ABC of Herbivory: How host plant toxins influence gene expression. Poster presented at 5th FEBS Special Meeting: ATP-Binding Cassette (ABC) Proteins: From Multidrug resistance to Genetic Diseases, Innsbruck, AT

Karpinski, Anne (2013). The ABC of drugs: How host plant toxins influence gene expression. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

Karpinski, Anne (2013). Detoxification processes in Bt resistant insects by ABC-transporters. Poster presented at 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, DE

Karpinski, Anne (2012). ABC transporters in insect detoxification pathways. Poster presented at 11th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Selbstständigkeitserklärung

Die derzeitig geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt. Die Dissertation mit dem Titel „ABC transporters in insect detoxification pathways“ wurde von mir eigenständig angefertigt. Es wurden keine Textabschnitte von Dritten oder eine meiner eigenen Arbeiten übernommen, ohne die entsprechende Kennzeichnung. Sämtliche Hilfsmittel und Quellen sind in der Arbeit genannt. Personen, die an der Gewinnung der Daten, sowie der Auswertung und der Erstellung der Manuskripte hilfreich waren, wurden namentlich gekennzeichnet. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Dritte haben für ihre Arbeit, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, keine geldwerte Leistungen erhalten. Die vorliegende Dissertation wurde weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht, noch als Dissertation an einer anderen Hochschule eingereicht.

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Anne Bretschneider

Jena, den 10.12.2015